Effect of salt stress on apoplastic barriers in roots and leaves of two barley species

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Declaration

I, hereby declare to have written this thesis independently without any help from others. All references and sources have been acknowledged specifically and used no other sources than those listed. This thesis has not been previously submitted for examination in any degree in this university or elsewhere.

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ABBREVIATIONS

ABA	Abscisic Acid
ABC	ATP-Binding Cassette Transporter
AHC	Alkyl Hydroxycinnamates
ANOVA	Analysis Of Variance
ASFT	Aliphatic Suberin Feruloyl Transferase
ATP	Adenosine Triphosphate
BF_3/MeOH	Boron Trifluoride Methanol
BLAST	Basic Local Alignment Search Tool
BSTFA	N, O-Bis (Trimethylsilyl)-Trifluoroacetamide
CBL	Calcineurin B-Like Protein
CIPK	CBL- Interacting Protein Kinase
CoA	Coenzyme A
CYP	Cytochrome P450 (CYP) Monooxygenases
DCA	Dicarboxylic Acid
DEGs	Differentially Expressed Genes
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
ETR	Electron Transport Rate
FAE	Fatty Acid Elongase
FAR	Fatty Acyl Reductase
FC	Fold Change
FDR	False Discovery Rate
FID	Flame Ionisation Detector
GAA	Gibberellic Acid
GC	Gas Chromatography
GO	Gene Ontology
GPAT	Glycerol 3-Phosphate Acyltransferase
HKT	High-Affinity Potassium Transporter

HPLC	High Performance Liquid Chromatography
HSP	Heat Shock Protein
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
KCS	B-Ketoacyl CoA Synthase
LACS	Long-Chain Acyl-CoA Synthetase
LSD	Least Significant Difference
LTP	Lipid Transfer Protein
MDA	Malondialdehyde
MDS	Multidimensional Scaling Plot
NIL	Near-Isogenic Line
NMR	Nuclear Magnetic Resonance
OSMOL	Osmolarity
PAM	Pulse Amplitude Modulation
PAR	Photosynthetically Active Radiation
PCR	Polymerase Chain Reaction
PEG	Poly Ethylene Glycol
PIP	Plasma Intrinsic Protein
PM	Plasma Membrane
PTFE	Polytetrafluoroethylene
qPCR	Quantitative Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SEA	Singular Enrichment Analysis
SOS	Salt Overly Sensitive
TIP	Tonoplast Intrinsic Protein
UV	Ultra Violet
VLCFA	Very-Long-Chain Fatty Acid
Y(II)	Quantum Yield Of Photosystem II

1. INTRODUCTION

1.1. Salt stress

Agricultural productivity plays a key role in compensating for the food demand in the rapidly growing human population. In vast fertile lands, food crops cultivations affected by climate change adversities chaperoned with biotic and abiotic stress pose a serious challenge. Stress is defined as an unfavourable environmental variable that affect the productivity and yield of plants. Living organisms have evolved to manage the extremities by either stress avoidance or stress tolerance. In plants, stress avoidance/tolerance are regulated by evolutionarily intricate molecular, genetic and biochemical means for effective stress acclimation response and management (Shanker and Venkateswarlu, 2011).

Salinity, characterized by a high concentration of soluble salts, is one of the major factors limiting the crop productivity quantitatively, by up to 25 % decrease in yield, and qualitatively in terms of germination and plant vigour (Munns and Tester, 2008). Soil salinity is the second most cause that leads to land degradation and an estimate of 2000 ha of arable land is affected daily globally (Zaman *et al*., 2018). More than 20 % of irrigated land have been affected by salinity (FAO, 2008; Meena *et al*., 2019). Even though inherently present salts contribute as an essential component of plant nutrients, the effect of bad irrigational practices (Munns, Goyal, and Passioura, 2005) along with natural accumulation over the time (Rengasamy, 2002) and also the deposition of oceanic salts by wind and rain pose drastic challenges for crops. Amongst various types of salts including chlorides of sodium, calcium and magnesium, the most soluble sodium chloride is found in abundance (Szabolcs, 1989). Mismanagement of salinity results in soil sodicity which deteriorate soil structure and eventually restricting soil aeration. As a result, plants will not only suffer from high salt toxicity but also hypoxia (Singh and Chatrath, 2001; Tisdale *et al*., 1993). Hence it is vital to address the challenges posed by salinity for efficient crop management.

Salt stress instantly affects the cell growth rate and subsequent accumulation of salt leads to toxicity and death of the tissue. Depending on the extent of exposure, salt stress can induce significant changes in physiological and metabolic levels. The effects associated with plant growth include reduced osmotic potential, nutrient imbalance and ion toxicity

(Ashraf, 1994). In the initial stage of salt stress, the water uptake by roots starts to decline with simultaneous water loss from the leaves. This deteriorating effect is due to the osmotic stress caused by the accumulation of Na^+ and Cl^- ions (Munns, 2005). Na^+ toxicity intervenes in significant cellular processes such as protein synthesis (Glenn *et al*., 1999), enzyme activity (Munns, 2002), chlorophyll production and photosynthesis (Zhani *et al*., 2012). Na^+ and Cl^- ions cause ion imbalance and interfere with the membrane transport of K^+ uptake which is vital for growth and development (James *et al*., 2011). According to Munns and Tester, 2008, plant response to salinity occurs in two phases: an osmotic and an ion-specific phase. In the osmotic phase, the rate of the young growing leaf is limited or delayed. However, in the ion-specific phase, salt tends to accumulate in older leaves at toxic levels. This eventually leads to leaf senescence, affects photosynthesis and consequently impedes the growth rate of developing leaves. Anatomical implications of salt stress were observed in the vascular tissue of mungbean seedlings (Rashid *et al.*, 2004). Other molecular level damage is rendered by Reactive Oxygen Species (ROS) such as H_2O_2, $HO\cdot$, $O_2^{\cdot -}$, which also cause secondary stress (Miller *et al*., 2010). ROS causes oxidative damage to cellular components such as proteins, lipids and even DNA and thereby resulting in impaired cellular functioning in plants (Gupta and Huang, 2014).

Plants are classified into glycophytes and halophytes depending on the manner it responds to salinity (Munns *et al*., 1983). Halophytes, throughout the years, have evolved salt tolerant mechanisms to survive and reproduce under high salinity conditions. These mechanisms could potentially be a key element in the development of more salt-tolerant crops. The mechanism of salt tolerance depends upon the severity of the stress. Under mild stress, salt tolerance is equated with the specific ion exclusion, whereas in a high salinity state, the salt-tolerant ability is correlated to the regulation of osmotic potential rather than ion exclusion (Tavakkoli *et al*., 2012). From literature, the ways plants adapt to salt stress can be broadly categorised into three levels – osmotic tolerance, Na^+/Cl^- exclusion from leaves and tissue tolerance. Osmotic tolerance mechanisms primarily include decreased leaf growth rate due to the osmotic effect of salt outside the roots. Prolonged exposure could also affect the flowering and maturation part of reproduction (Munns and Tester, 2008). As mentioned earlier, a reduced photosynthesis rate leads to the development of ROS. This is

due to the changes in leaf morphology, chlorophyll pigmentation and inactivity of antioxidant enzymes in the presence of salt (Apel and Hirt, 2004).

The initial realisation of salinity by roots will trigger immediate long-distance signalling to shoot partly mediated by ABA. However, in the event of water deficit, hydraulic responses precede the ABA signalling from the roots (Christmann *et al*., 2007). Nevertheless, abiotic stresses including drought and salinity lead to increased concentration of ABA accompanied by genetic and physiological responses. These responses are important for the adaptation to high salt levels (Knight *et al*., 1997; Rabbani *et al*., 2003; Tester and Leigh, 2001; Tracy *et al*., 2008). Another critical response is the increase of cytosolic Ca^{2+} levels (Kiegle *et al*., 2000) which are involved in the activation of calcineurin B-like protein (CBL4) previously designated as SOS3 (salt overly sensitive). Cytosolic Ca^{2+} also facilitates the dimerization of CBL4/SOS3 followed by interaction with CBL- interacting protein kinase (CIPK24) also known as SOS2. This CBL4-CIPK24 (SOS3/SOS2) complex mediates the phosphorylation and subsequently activates Na^+/H^+ antiporter (SOS1). SOS1 or Na^+/H^+ antiporter is responsible for Na^+ extrusion and its activation is solely dependent on SOS2 and SOS3 (Halfter *et al*., 2000; Qiu *et al*., 2002; Shi *et al*., 2002). Based on these findings, a proposed model states that the management of ion balance in a high Na^+ environment by SOS genes is facilitated by (i) Na^+ extrusion from the cell via Na^+/H^+ antiporter pump, (ii) vacuolisation of Na^+ via tonoplast transporters and (iii) alteration of root architecture by PIN-dependent auxin transport **(Figure 1)** (Ji *et al*., 2013).

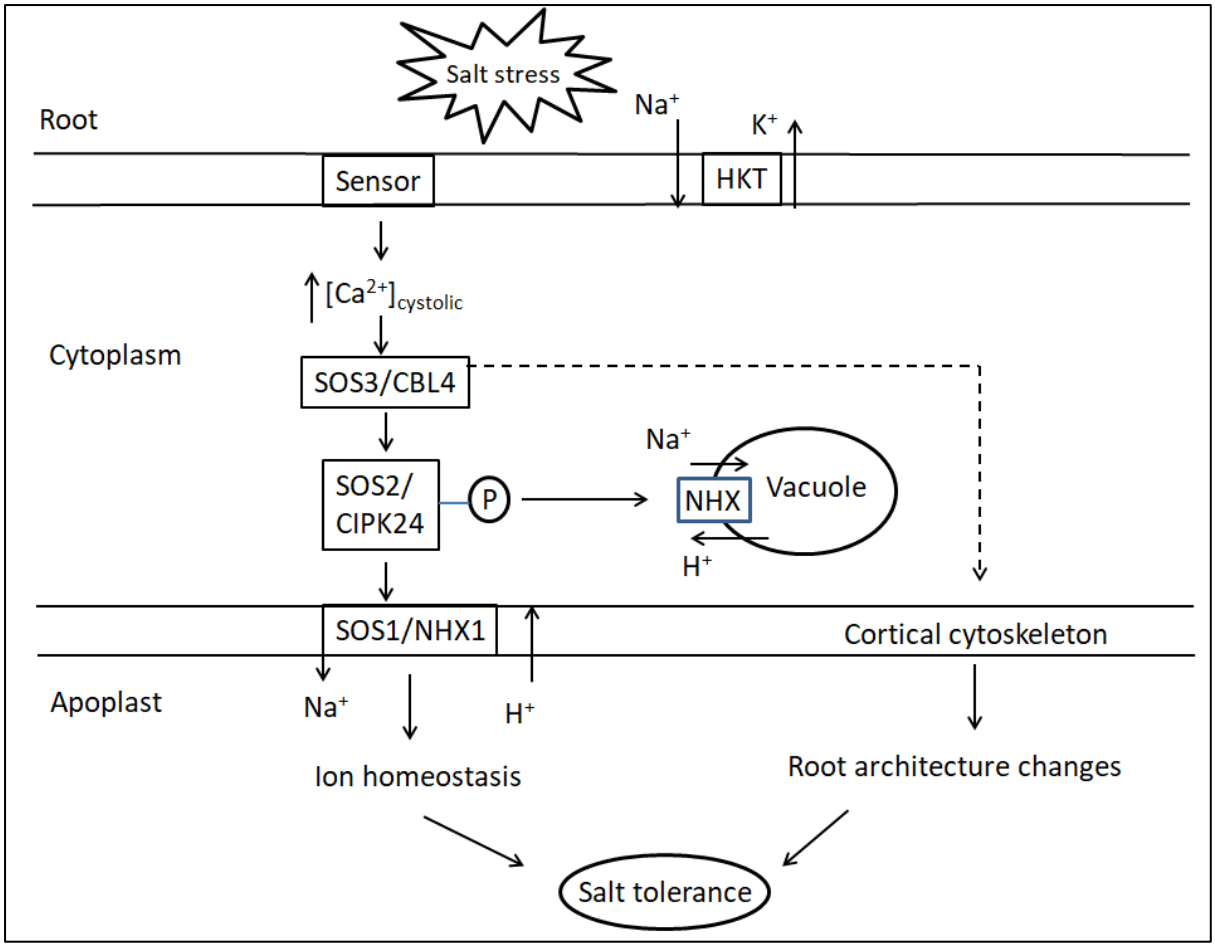

Figure 1- Salt tolerance mechanism via SOS signalling pathway

The model of SOS signalling mechanism in the root cells to confer ion homeostasis, root architectural changes such as lateral root development, and other cellular level processes in response to salt stress. Solid arrows indicate established, direct regulations, while dashed lines indicate suggested links between the components represented (modified from Ji *et al.,* 2013).

Studies on the *sos* mutants of *Arabidopsis thaliana* showed reduced tolerance to salinity compared to the wild types (Zhu *et al*., 1998). In wheat, supplemental calcium enhances salt tolerance, particularly in sensitive genotypes (Genc *et al*., 2010). Apart from SOS, other transporters like HKT, NHX play important role in sodium efflux and compartmentalisation (Zhu, 2002). To alleviate the effect of salt stress, plants tend to accumulate organic solutes such as linear polyols (glycerol, mannitol or sorbitol), cyclic polyols (inositol, pinitol and/or derivatives), amino acids (glutamate or proline) and betaines (glycine betaine or alanine betaine) (Flowers *et al*., 1977; Hasegawa and Bressan, 2000; Munns, 2005). These organic solutes even at high concentrations cause no harm to the plants and are only involved in osmotic adjustment and henceforth they are also referred as compatible osmolytes (Zhu, 2007).

Barley (*Hordeum vulgare* L.), one of the earliest domesticated crops since the dawn of agricultural practices, belongs to the family *Poaceae*, tribe *Triticeae* and genus *Hordeum*. Due to its enormous adaptability to adverse climatic and soil conditions, it is considered one of the prominent food crops all around the world. The wild relative of barley is identified to be *Hordeum spontaneum* C. Koch. According to modern taxonomy, *Hordeum vulgare* L. and Hordeum *spontaneum* C. Koch. are categorised to be the subspecies of *Hordeum vulgare* (Badr *et al*., 2000). The geographic habitat of ancient cultivation of the wild progenitor, *H. spontaneum* spans from Israel and Jordan to south Turkey, Iraqi Kurdistan to southwestern Iran (Harlan and Zohary, 1966; Nevo, 1992). Further exploration indicates colonization by *H. spontaneum* in south-east Iran, Afghanistan and the Himalayan belt (Zohary, 1993). Additional mutations accompanied by spontaneous hybridization between different barley cultivars and also *H. spontaneum* resulted in the divergence of morphological and physiological traits. These allelic variations act as a subjective force for natural selection to acclimatize adverse/unfavourable growth conditions (Slafer *et al*., 2002). Apart from being an important agricultural crop, the cultivar *Hordeum vulgare* L. is employed as a model organism in the genetic studies of angiosperms. Due to its high self-fertility rate, low number of chromosomes (2n=14), ease of hybridization, large chromosome which facilitates convenient observation on chromosomal anomalies and simplified hereditary trait classification, it is extensively used as a chief model among the higher plants (Nilan, 1974).

Barley is one of the most salt-tolerant crops and exhibit varying levels of tolerance among different cultivars and also between the wild (*Hordeum spontaneum*) and cultivated barley (*Hordeum vulgare*). Studies show that the salinity affects the seed germination rate of Iranian barley cultivars significantly and followed by reduced shoot and root lengths (Movafegh *et al*., 2012). Short term growth responses of barley leaves showed that the changes in water potential gradient after the addition of salt solution spontaneously decreased the leaf elongation velocity and recovered as early as 30 minutes later. Further parameters such as rate of transpiration, ABA and cytokinins levels, water channel activity and cuticular wax deposition were also investigated (Fricke *et al*., 2006). Chlorophyll content, proline, water-soluble carbohydrate concentrations were also affected by the salinity (Movafegh *et al*., 2012).

Several studies have had been conducted on one of the main aspects of salt tolerance *per se*, Na^+ ion accumulation and extrusion. Na^+ accumulation with a decline in K^+; differential concentration of malondialdehyde concentration (MDA) and hydrogen peroxide (H_2O_2) in Egyptian barley *(Hordeum vulgare* L.) cultivars were found (Elsawy *et al*., 2018). Another study on the sensitive and tolerant cultivar of barley named Triumph and Gerbel respectively proposed that the translocation of ions to the shoot depends on cytosolic ion concentrations and that the accumulation of Na^+ was higher in sensitive variety (Triumph) than the tolerant cultivar (Gerbel) (Flowers and Hajibagheri, 2001). Nassery and Baker, 1974 proposed that the Na^+ extrusion pump in barley roots efficiently function at salt concentrations above 50 mM and prevents Na^+ entry in the presence of potassium and calcium.

Proteomic analysis on salt-stressed barley identified proteins associated with growth regulatory mechanisms, membrane stability (Witzel *et al*., 2014), ion homeostasis (via cell membrane transporters), metabolic processes and (Wu *et al*., 2014). Furthermore, proteins involved in glutathione-based detoxification of reactive oxygen species (ROS) (Witzel *et al*., 2009) were enriched. Genetic studies on wild barley leaves indicated high-level expression of transcripts related to electron transport, flavonoid biosynthesis and ROS scavenging activity (Bahieldin *et al*., 2015). RNA sequencing of near-isogenic lines (NIL) of Chinese Landrace barley variety TX9425 containing Quantitative trait loci (QTL) related to salt tolerance on chromosome 2H revealed potential candidate genes such as HSP90 (heat shock proteins) and signalling related receptor-like kinase (Zhu *et al*., 2020). Another study showed that the genes in barley near-isogenic lines (NILs) involved in metabolic pathways such as suberin, cuticular wax and phenylpropanoids biosynthesis were differentially regulated (Glagoleva *et al*., 2017). Ho *et al*., 2020 proposed distinctive salinity adaptations of apoplastic barriers in different barley genotypes that facilitate sustainable growth and salt toxicity. Combined studies of salinity with drought stress indicated different transcript profiles possibly for similar functions essential for adapting to multiple abiotic stress (Ozturk, 2002).

1.2 Suberin- Location and function

Cell walls of plants have specialized lipid-phenolic based barriers that play a pivotal role in water and solute movement, wound healing and also in adapting to various abiotic stress environments such as drought, salinity, high temperatures etc., (Franke and Schreiber, 2007; Schreiber, 2010). Major constituents of the apoplastic barriers are suberin, lignin and cutin. Suberin and lignin are secondary cell wall modifications in the epidermis and the periderm (Kumar *et al*., 2016) that enhance structural integrity (Kolattukudy, 2001) and protect plants in the event of pathogen attack (Nawrath, 2002). Unlike lignin and suberin, cutin barriers are found on epidermal leaf cuticles (Bourgault *et al*., 2020), fruits, flowers and also seed coats (Espelie *et al*., 1979).

Suberin, a glycerolipid - phenolic extracellular heteropolymer is primarily deposited in the root endodermis and seed coat (Vishwanath *et al*., 2015). The inducible factors for suberin production include environmental stresses such as desiccation exposure, high salt concentration, wounding, pathogenic microbial invasion and heavy metal pollution in the soil (Krishnamurthy *et al.*, 2011; Ranathunge, Schreiber and Franke, 2011). Other factors such as temperature extremities, osmotic shock and soil acidity are also contributory to suberin deposition (Kolattukudy, 2001; Franke, Dombrink and Schreiber, 2012).

Suberin deposition is pronounced in the form of Casparian bands and suberin lamellae. Casparian bands span the space between the endodermal cell walls adjacently and have lignin as a prominent constituent (Naseer *et al*., 2012; Schreiber, 1996) with some level of suberin (Zeier and Schreiber, 1998). It acts as a primary barrier to the apoplastic movement of molecules (Nawrath, 2002; Hosmani *et al.*, 2013) and controls the movement of essential ions such as NaCl within the root cells (Ranathunge and Schreiber, 2011). Suberin lamellae are present as a single or multi-layered deposition that is radially arranged along the endodermal cell walls (Enstone *et al*., 2002). Suberin is also deposited in seed coats (Molina *et al*., 2008). Suberized cell walls act as highly hydrophobic barriers and contribute to the impermeable nature of the seed coat during maturity (Boesewinkel and Bouman, 1995). Suberized cells contribute to form wounding tissue post-physical injuries in plants and also acts as a sealing layer to facilitate the abscission of plant parts (Van Doorn and Stead, 1997).

1.3 Structure of suberin

Suberin, a lipophilic heteropolymer constitute of long-chain poly aliphatic polyester interlinked with phenolic compounds and wax enclosures (Franke and Schreiber, 2007; Kolattukudy, 1981; Pollard *et al*., 2008). The aliphatic units of suberin are identified to be α,ω-dicarboxylic acids, ω-hydroxyl fatty acids, primary fatty alcohols and unsubstituted fatty acids (Kolattukudy, 2001; Pollard *et al*., 2008).

Suberin acids are α, ω-bifunctional molecules containing functional groups at both ends of the hydrocarbon chains. Based on this arrangement, they are classified as α, ω-diacids with carboxyl groups at both α and ω positions; ω- hydroxyl fatty acids with a carboxyl group at α and a hydroxyl group at the ω position. These two linking positions are crucial for the polymeric crosslinking of suberin monomers. α,ω- diacids and ω-hydroxyl fatty acids account for 80-90% of the total aliphatic content whereas monofunctional fatty acids and fatty alkanols are present < 10% of the overall amounts (Graça, 2015).

The identified chain lengths of α,ω- diacids and ω- hydroxyl fatty acids range between C_{16}-C_{30} (Holloway, 1983; Krishnamurthy *et al*., 2009) with the even number chain lengths predominantly at higher concentrations (Kolattukudy, 2001). C_{18} units of both α, ω-diacids and ω-hydroxy acids have been found to have mid-chain modifications and saturated chains (starting at C_{16} and goes up to C_{28}). The mid-chain modifications are categorised into three types and are found at the C_9 and C_{10} positions. The mid-chain groups can be modified through a double bond, an epoxy group and two vicinal hydroxyl groups (Santos *et al*., 2013). These secondary groups significantly affect the complexity of the suberin polymer arrangement (Graça, 2015). Non-soluble polyphenolic compounds termed “polyaromatics” constitute 25% of the suberin structure in cork (Pereira, 1988) and 31% in potato periderm (Mattinen *et al*., 2009).

1.4 Suberin biosynthesis

Suberin accumulation in the plant tissue requires sequential synthesis of aliphatic and aromatic subunits, transportation to cell walls to form non-soluble assembly via controlled polymerization. However, the underlying phenomenon of these mechanisms is not yet fully understood.

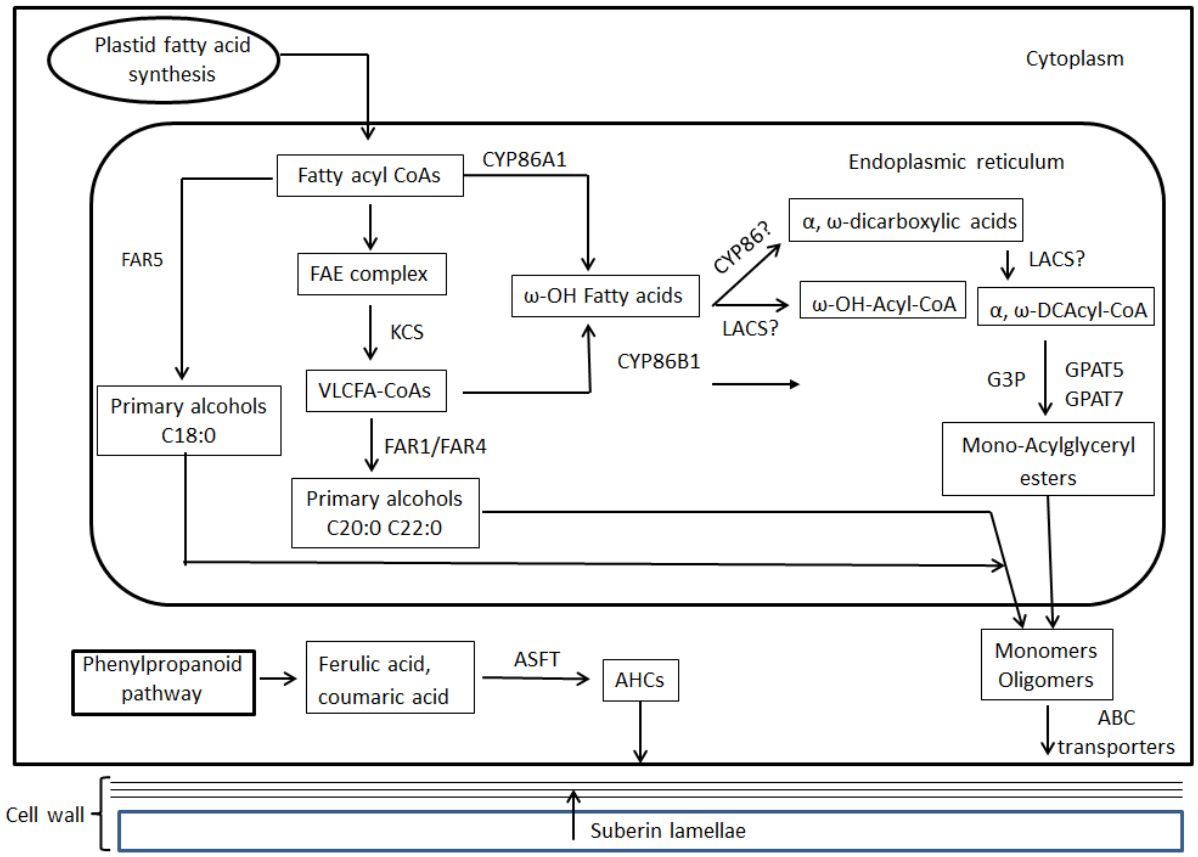

Figure 2- Overview of suberin biosynthetic pathway

Plastid fatty acid synthesis results in fatty acyl CoAs and is exported into endoplasmic reticulum. Suberin biosynthetic enzymes aid in the synthesis of monomers. Fatty acyl elongation happens through Fatty Acid Elongation complex (FAE) which produce very long chain fatty acids (VLCFA).Fatty Acyl Reductsae (FAR) aids in the synthesis of primary alcohols and α, ω diols.Cytochrome P450 enzymes (CYPs) helps in the formation of ω-hydroxy fatty acids (ω-OHs) and α, ω dicarboxylic acids (DCAs). CYP86A1 is involved in the synthesis of short chained ω-OHs whereas CYP86B1 is involved in the long chain ω-OHs. Glycerol 3-phosphate acyl transferases (GPATs) are involved in the esterification of ω-OHs and DCAs to glycerol 3-phosphate (G3P). Long chain acyl-CoA synthetases (LACSs) catalyse fatty acid activation to Fatty acyl CoAs. ASFT- Aliphatic Suberin Feruloyl Transferase. The aromatic units such as coumaric and ferulic acids are synthesised through phenylpolypropanoid pathway and are associated to fatty alcohols by BAHD-type acyl transferase to form alkyl hydroxycinnamates (AHCs).Mechanisms involved in transport and polymerization is still ambiguous. ATP binding cassette (ABC) transporters support the transport of monomers across the plasma membrane (modified from Vishwanath *et al*. 2015).

The free fatty acids C_{16} and C_{18} carboxylic acids, derived from the plastidic fatty acid biosynthesis (Franke *et al*., 2005) are converted into fatty acyl CoA thioesters by long-chain acyl-CoA synthetases (LACSs) (**Figure 2**). Following the acyl activation, the elongation of fatty acyls is activated by a set of suberin synthesis enzymes localized in the endoplasmic reticulum. This enzyme complex termed as fatty acid elongase (FAE) complex comprises four enzymes (Samuels *et al*., 2008). The elongation produces long-chain and very long-chain

fatty acids, characteristic of the suberin polymer. The extent of elongation is scrutinised by β-Ketoacyl CoA synthase (KCS) which is the primary enzyme in the fatty acid elongase unit (Millar and Kunst, 1997).

Hydroxylation of the fatty acids at the terminal methyl position (ω) by CYP86 (a subfamily of cytochrome P450 (CYP) monooxygenases) results in the synthesis of $C_{16:0}$ and $C_{18:1}$ ω-hydroxyl fatty acids (ω-OHs) (Molina *et al*., 2009). A small proportion of this is oxidized to dicarboxylic acids (α,ω-DCAs) by ω-hydroxyl fatty acid dehydrogenases (Agrawal and Kolattukudy, 1978; Kurdyukov *et al*., 2006). Two cytochrome P450 dependent fatty acid oxidases and hydroxylases involved in Arabidopsis root suberin synthesis are identified to be CYP86A1 and CYP86B1 (Compagnon *et al*., 2009; Höfer *et al*., 2008; Li *et al*., 2007; Molina *et al*., 2009). CYP86A1 is responsible for synthesizing ω-hydroxyl acids of chain length varying between C_{12} and C_{18}. This process is critical for the earlier deposition of suberin in the primary endodermis (Höfer *et al*., 2008). CYP86B1 is involved in producing long-chain ω-hydroxyl acids with a length between C_{22}-C_{24} (Compagnon *et al*., 2009; Molina *et al*., 2009). Saturated primary alcohols of chain length C_{18}, C_{20} and C_{22} (Schreiber *et al*., 2005) constitute 6-7% of total aliphatic suberin (Pollard *et al*., 2008). They are produced by the reduction of fatty acids via fatty acyl reductase (FAR) (Domergue *et al*., 2010). The aliphatic and phenolic components of suberin are covalently bound to glycerol (Graça and Pereira, 2000; Moire *et al*., 1999).

Monoacylglycerols, considered to be initial building blocks of suberin biopolymer are generated by esterification reactions mediated by acyl CoA dependent glycerol 3-phosphate acyltransferases (GPATs) (Yang *et al*., 2012). The aromatic fractions are hydroxycinnamates such as ferulic and coumaric acids and are synthesized as the products of the phenylpropanoid pathway. The aromatic units are covalently linked with aliphatic domains through ester linkage (Bernards *et al*., 1995).

The transport of aliphatic suberin constituents synthesized in the endoplasmic reticulum is speculated to occur via a secretory pathway. It has been established that Golgi and *trans* Golgi network-mediated vesicular trafficking are involved in the export of cuticular waxes to the apoplasm (McFarlane *et al*., 2014) and export of suberin precursors through secretory vesicles. The role of ABC transporter in exporting aliphatic suberin components in

Arabidopsis thaliana was studied extensively (Shanmugarajah *et al*., 2019); however, the substrate specificity of aliphatic and aromatic units in these families of transporters is yet to be identified. Lipid transfer proteins (LTPs) are another set of transporters that might be involved in suberin export. *KO* mutants of these proteins in *Arabidopsis thaliana* showed reduced suberin deposition (Open *et al*., 2016). It has been proposed that LTPs assist ABC transporters in export and eventually facilitate the transport of suberin monomers to the site of deposition due to their hydrophilic nature (Edqvist *et al*., 2018).

1.5 Objectives

Salinity is one of the most important constraints affecting the crop production worldwide. It is vital to understand the salt stress responses and tolerance behaviour and subsequently apply this knowledge to effectively develop salt stress resistant plants in the longer run. To attain this goal, halophytes such as barley can be studied to identify traits that could potentially help in conferring salt tolerance especially in glycophytes such as rice.

In this study, the outcome was mainly aimed to understand the differential responses exhibited due to salinity and also to investigate the variability in salt stress specific responses between cultivated barley (*Hordeum vulgare* L. spp.*vulgare)* cv Scarlett and a wild accession (*Hordeum vulgare* spp. *sponataneum*) from Pakistan (ICB181243). Previous study by Kreszies *et al*., 2019 and 2020 witnessed differential behaviour between Scarlett and wild barley of Pakistan accession in response to osmotic stress. Hence, this study was aimed to see how Scarlett and Pakistan wild barley behaves in the event of salt stress. Salt concentrations were intended to attain water potentials of -0.4,-0.8 and -1.2 MPa and were ranging from mild (80 mM) to moderate (180 mM) and severe (275 mM) stress concentrations.

The primary response of plants during salt stress is by limiting their growth. Another critical response is the development of suberized barriers, mainly to limit the influx of solutes and specifically to limit Na^+ entry into the xylem. While it is not possible to completely filter out Na^+ from the growth medium in entering the roots, halophytes have the capability to translocate the ions from the root to the shoot. This transport leads to the accumulation of ions within the leaves which in turn affects the photosynthesis apparatus and also triggers the synthesis of biological solutes to aid in the osmotic adjustments. In order to study this cascade of responses under varying degree of salinity, the experiments were divided into three parts:

- The effect of salt stress on apoplastic barriers in roots (suberin) and leaves (wax and cutin) were analytically investigated using GC-MS/FID and the suberin development in roots were histochemically examined.
- RNA sequencing was done in root segments that correspond to 0-12.5 % of the length of the barley roots.

- Physiological experiments included measurement of osmotic potentials within roots, Na^+ accumulation and ICP analysis of ions, proline accumulation in leaves and roots, stomatal conductance and photosynthetic yield in leaves.

Results obtained in these experiments could provide more information how barley adapts and manages salt stress. In addition, this approach could show whether responses of wild barley are different compared to cultivated barley.

2. MATERIALS AND METHODS

2.1 Plant material and growth condition

Barley seeds of cultivar Scarlett and wild type ICB181243 (wild accession from Pakistan) were germinated in wet paper towels for three days at 25˚C in dark. Since the wild accessions have a longer dormancy, 1-2 ml of 150mM gibberellic acid (3-GAA) was added to stimulate germination. Three-day old seedlings were hydroponically grown in pots (3.5 L) with half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938) with continuous air supply. Growth room conditions were 16 h light and 8 h dark at day/night temperatures of 23˚C and 20˚C and light intensity of 130 µm m^{-2} s^{-1}. The relative air humidity was between 50-65%. The total growth period was twelve days and at that stage, the plants had developed two leaves and five to six seminal roots.

2.2 Salt stress application

Salt stress was applied six days after the germination of barley seeds. Half strength Hoagland nutrient solution was complemented with 80 mM, 180 mM and 275 mM NaCl. Osmolarity and water potential of the nutrient solution with the salt were measured using Osmomat O30 Cryoscopic osmometer (Gonotec GmbH, Berlin, Germany) and WP4C dew point hygrometer (Decagon device, Meter Group Inc., Pullman, WA, USA) respectively (**Table 1)**.

Table 1 - Water and osmotic potential values for different solution mixture

	Water potential ψ(MPa)	**Osmotic potential (osmol.kg^{-1})**
Control	0.02	0.02
80 mM	- 0.4	0.17
180 mM	- 0.8	0.35
275 mM	- 1.2	0.50

2.3 Harvest of roots and zone segregation

Twelve-day old seedlings were harvested and the root and shoot length were measured. The seminal roots of barley were then categorised into different zones depending on relative suberin deposition based on the observations done previously by Kreszies *et al*. 2019. The harvested roots were further used for histochemical, analytical, RNA sequencing, proline analysis and elemental variation analysis.

2.4 Histochemical assessment

Barley roots were histochemically examined along the length for the detection of suberin lamellae and Casparian bands. Before microscopy, the harvested roots were immersed in fixation solution (**Table 2**) to keep the root intact and to maintain its integrity during the storage period.

Table 2 - Composition of the fixation solution

Disodium hydrogen phosphate (Na_2HPO_4)	10 mM
Sodium chloride (NaCl)	137 mM
Potassium chloride (KCl)	2.7 mM
pH adjusted to 7.4	
Formaldehyde (CH_2O)	3.7% (v/v)

2.4.1 Preparation of the slide

Roots of approximately 1 cm length were placed in moulds containing NEG 50 (Richard-Allen Scientific, Thermo Fisher Scientific Inc.), a colourless viscous liquid soluble in water. From the frozen root sample, thin cross-sections (40 µm) were made with the microtome (Microm HM 500M, Microm International, Walldorf, Germany). The cross-sections were carefully transferred to holders with a microsieve filter of pore size less than 10 µm and washed carefully with water to remove the NEG. The holders were then incubated in the corresponding staining solution for the selective observation in the epifluorescence microscopy with Zeiss AxioPlan microscope (Carl Zeiss, Germany) under UV light. The images were photographed with a mounted Canon EOS 600D SLR camera (Canon Inc. Tokyo, Japan).

2.4.2 Fluorol Yellow 088 (Suberin lamellae) staining

Fluorol yellow 088 staining was performed to visualize suberin lamellae. This dye specifically stains lipid structures bright yellow and imparts contrast on suberin lamellae (Brundrett *et al*., 1991).

Table 3 - Fluorol Yellow 088 composition

Fluorol yellow 088	0.01 % (w/v) in PEG 400
Glycerol	90% (v/v)

Fluorol Yellow 088 (0.01% w/v) was dissolved in PEG 400 by heating at 90˚C for 1 h and an equal volume of 90% (v/v) glycerol was added. The cross-sections of roots were placed in section holders containing the staining solution for 1 h at room temperature in dark. The cross-sections were rinsed with water several times and transferred to the object slide. The slide was observed under UV light. Care was taken to keep the exposure time as short as possible due to the fading of stain at UV illumination.

2.4.3 Berberine-Aniline Blue (Casparian bands) staining

Berberine –Aniline staining distinguishes the Casparian bands in root exodermal and endodermal cells. Berberine confers non-specific staining across the cells. Aniline Blue acts as a counterstain and facilitates the visualization of Casparian bands by partially quenching the background fluorescence and non-specific staining by Berberine. This phenomenon induces a greater colour contrast between the aniline counterstained (blue-yellow) and berberine stained (yellow) structures in the pictures (Brundrett *et al*., 1988).

Table 4 - Berberine-Aniline Blue dye composition

Berberine hemisulphate	0.1 % (w/v)
Aniline blue	0.5% (w/v)
Ferric chloride ($Fe\ Cl_3$)	0.1% (w/v)
Glycerine	50% (v/v)

The cross-sections were stained with 0.1 % (w/v) berberine for 1 h at room temperature and washed with water to remove the staining solution and incubated with 0.5% aniline blue for 30 minutes. The section holders were rinsed with water several times and transferred to slides to observe under UV light.

2.5 Chemical analytics of suberin in roots

Seminal roots of both Scarlett and wild barley of Pakistan accession were segregated as elaborated in section **2.3**. Suberin amounts (µg) were referred with respect to the endodermal surface area (cm^2). The endodermal area was calculated for each root zone using the formula, A= 2 πrL (r, endodermis radius; L, length of the individual root zone). For each replicate, 10 segments were pooled for each zone per treatment. Three biological replicates were used for each experiment. The segments of the seminal roots were further subjected to enzymatic digestion before the analysis by gas chromatography.

2.5.1 Sample preparation and suberin extraction

Enzymatic cell wall isolation

A typical plant cell wall comprises proteins, aromatic components, major carbohydrates such as cellulose, pectin, lignin and hemicelluloses (Caffall and Mohnen, 2009). To disintegrate these constituents, the harvested root segregates were immersed in an aqueous solution of pH 3 containing 0.5% (w/v) cellulase and pectinase along with 1mM NaN_3 to prevent microbial growth (**Table 5**). This leads to the separation of endodermal and hypodermal cell walls (Schreiber *et al*., 1999). The root segments within the solution were vacuum infiltrated to facilitate effective interaction between the enzyme solutions and roots and was continuously shaken. The solution was changed every 3-5 days for 3 weeks (Schreiber *et al*., 1994).

Table 5 - Composition of the enzyme solution

Enzyme solution	
Citric acid (pH-3)	10 mM
Cellulase	0.5% (w/v)
Pectinase	0.5% (w/v)

Sodium azide (NaN_3)	1 mM
Borax buffer	
Sodium tetraborate pH-9	0,01 M
Chloroform - methanol solution	
Chloroform	50% (v/v)
Methanol	50% (v/v)

Following the enzymatic treatment, borax buffer (pH 9) was added to remove phenolic compounds. After two days, the root fragments were carefully suspended in chloroform/methanol solution (1:1) to remove soluble lipids (Simone *et al*., 2003). The solution was changed every three days for a week. As final step roots samples were dried on polytetrafluoroethylene (PTFE) over activated silica gel in a desiccator. Glass vials used for analytics were cleaned with chloroform by placing them horizontally on a rolling bench apparatus (CAT RM 5 – 30 V, Staufen, Germany) for more than 20 minutes at 100 rpm. The chloroform was then dispensed and the vials were completely dried. The dry weights of the samples were determined accurately using the Sartorius MC 21S weighing balance (accuracy ± 1 µg) and a maximum of 5 mg were used for the analysis.

Transesterification with BF_3/MeOH

Transesterification of root samples released the suberin monomers. Treatment with BF_3/MeOH resulted in aromatic monomers alongside esterified long-chain fatty acid derivatives like ω-hydroxy acids and α,ω-dicarboxylic acids that constitute the typical suberin polymer (Zeier and Schreiber, 1997). Transesterification or ester exchange begins with the initial protonation of ester followed by the replacement with alcohol.

The samples were transesterified in a 4-9 ml vial with 1-2 ml BF_3/MeOH (Boron trifluoride methanol) for 16 h at 70˚ C in the heat block. The vials were removed from the heat block after 16 h and cooled down to room temperature. 50 µl of internal standard dotriacontane (C_{32} alkane) from the standard stock solution (10 mg dissolved in 50 ml) was added to each trans-esterified solution and vortexed. The transesterification reaction was

stopped by transferring the samples to another vial containing 2 ml of saturated $NaHCO_3/H_2O$ (sodium bicarbonate) slowly to prevent the formation of gas bubbles.

Extraction with chloroform

Chloroform of volume 1-2 ml was added to the vials with samples and vortexed. This enables the phase separation and the lower phase was transferred into clean glass vials. This step was repeated twice. Glycerol, a polar suberin monomer was not extracted with chloroform and thus remained in the aqueous phase. The extracts were washed with 1-2 ml of HPLC water. The upper phase was discarded and the extracts were dried with anhydrous Na_2SO_4 (sodium sulphate). The extracts were transferred to the reaction vials and were up concentrated by evaporation under a nitrogen stream at 60˚ C.

Derivatisation of the extracts

Monomers obtained after transesterification and chloroform extraction were derivatised by N, O-Bis (trimethylsilyl)-trifluoroacetamide to convert free hydroxyl and carboxyl groups to their respective trimethylsilyl derivatives. The derivatisation catalyzed by pyridine confers thermal stability and volatility to the sample, thus making it ideal for GC analytics (Simone *et al*., 2003). 20 µl of pyridine and BSTFA (N, O-Bis (trimethylsilyl)-trifluoroacetamide) were added to the react vials for derivatisation of the samples followed by incubation at 70˚C for 40 minutes in the heating block.

2.5.2 Gas chromatography analysis (GC-MS/FID)

Gas chromatography was employed for quantitative and qualitative suberin analysis. GC-Mass spectrometry was used qualitatively to identify the compounds characteristic of suberin whereas GC- Flame Ionization Detector quantitatively assessed the concentration of the suberin monomers.

GC-MS (Mass Spectrometry- Agilent technologies, 7890B/5977A Series Gas Chromatograph/Mass Selective Detector) has a quadruple mass analyzer that identifies the compounds based on mass to charge ratio. The signal peaks on the chromatogram were identified by comparing it with the database established in Prof. Schreiber's laboratory (Department of Ecophysiology, Institute of Cellular and Molecular Botany, Bonn, Germany)

and further elucidation was assisted by Agilent software - GC/MSD Mass Hunter Acquisition with both Mass Hunter and Classic Chem Station Data Analysis.

Table 6 - Temperature profiles of GC-MS for the analysis of suberin

GC MS	Temperature rise (˚C/min)	Final temperature (˚C)	Temperature hold (min)
Suberin		50	2
	45	200	1
	3	300	15

GC-FID (Flame Ionization Detector- Agilent technologies, 6890N Network Gas Chromatography) was used to quantify the suberin monomers from the peak area in the chromatograms. The instrument uses hydrogen flame as a carrier gas and has a column length of 30 m and 0.32 mm diameter. The column was coated with 0.1 µm poly (dimethylsiloxane). The derivatised sample of volume, approximately 200 µl was transferred to autosampler vials. Upon the injection of 1 µl of sample, the compounds within the sample disintegrate into free ions which will be interpreted as an electric signal at the outlet.

Table 7 - Temperature profiles of GC-FID for the analysis of suberin and acid standard

GC FID	Temperature rise (˚C/min)	Final temperature (˚C)	Temperature hold (min)
Suberin		50	2
	10	150	1
	3	310	20
Acid standard		50	1
	40	200	2
	3	310	20

To evaluate the quality of the column after the analysis, an acid standard solution containing alkane (C_{24}) and three carboxylic acids (C_{29}, C_{30}, C_{31}) in chloroform was used. Before use, the solution was derivatised and an appropriate temperature profile (**Table 7**)

was adapted for the acid run. From the resulting chromatogram, the ratio of area concentration for alkane to that of C_{31} was calculated. The ratio should be ≤ 1.3 for the column to be considered with sufficient quality for future analysis.

2.6 ICP elemental analysis

The analysis was done in Plant Nutrition and Crop Physiology Department, Georg-August-Universität Göttingen, Germany. The samples were prepared and analysed as described in Tränkner and Jaghdani, 2019. Using a high-accuracy balance, 100 mg of dried and powdered plant material was transferred to a Teflon digestion tube. The digestion medium consisted of 4 ml concentrated HNO_3 and 2 ml 30% H_2O_2 and micowave-digestion was performed at 200 °C at 15 bar for 75 min (Ethos.lab, MLS, Germany). After digestion, samples were diluted in 25 ml double-distilled H_2O. In each batch of microwave digestion, a certified reference material (apple leaf, SRM 1515, National Institute of Standards and Technology, USA) was also digested. Ion concentrations were measured by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Vista RL, CCD simultaneous ICP-OES, Varian Inc., USA) equipped with a Quarz Torch Low Flow with a 1.4 mm injector and a Sea Spray nebulizer with sample uptake of 2 ml min^{-1}. Calibration was achieved by a multielement standard solution purchased from Bernd Kraft, Germany. After approx. each 20 samples, measurement of the certified reference material is included to ensure accuracy of measurements. For this analysis, 12d old barley leaves and roots grown in control and 80 mM NaCl were used and aat least three biological replicates were used for data acquisition.

2.7 Determination of proline

The concentration of proline in the root and shoot of salt-stressed barley was assessed. The plant material was frozen in liquid nitrogen, followed by the pulverization by mixer mill (Retsch MM400; Retsch GmbH, Haan, Germany) at a frequency of 30 rounds s^{-1} for 1 min. The powdered samples (approx. 100 mg) were subjected to proline extraction as per the ninhydrin method adapted from Bates, 1973. The amount of proline (µg.g^{-1} of fresh weight) was determined photometrically at the absorbance of 520 nm. Three biological replicates were used for each measurement.

2.8 Estimation of osmotic potential in roots

The measurement was done as per the protocol described in Kreszies *et al*. 2019. Five seminal roots were grounded using a mixer mill (Retsch MM400; Retsch GmbH, Haan, Germany) at a frequency of 30 rounds s^{-1} for 1 min. The samples were centrifuged at 10,000 rpm for 2 min. The concentration of the supernatant was measured with an osmometer (Gonotec Osmomat 030; Gonotec GmbH, Berlin, Germany). The resulting concentration in mOsmol·kg^{-1} was converted to osmotic potential using the van't Hoff equation: $\Psi = MiRT$ with M = concentration in molarity, i = van't Hoff factor, R = rate gas constant, T = absolute temperature (K). Three biological replicates were used for each experiment.

2.9 RNA sequencing

RNA sequencing was done for 12 d old barley roots stressed with 180 mM NaCl which was equivalent to the water potential of –0.8 MPa. Only 0-12.5 % of Zone A was used to sequencing.

2.9.1 Sample preparation and RNA isolation

The root samples were collected in 2 ml sterile Eppendorf tubes and were instantly frozen in liquid nitrogen. Around 10 roots were pooled for each treatment and were powdered using sterile metal beads with the help of a mixer mill (Retsch MM400; Retsch GmbH, Haan, Germany) at a frequency of 30 rounds s^{-1} for 1 min. From the powdered sample, RNA isolation and extraction was done as per the protocol described in RNeasyPlus Universal Mini Kit (Qiagen, Venlo, Netherlands). The integrity of the RNA was determined through Nanodrop (Thermo Fischer Scientific, Wilmington, Delaware, USA) Agilent RNA 6000 Nano Chip (Agilent Technologies, Santa Clara, CA, USA) Bioanalyzer. Sample with a RIN value of ≥ 6.0 was used for sequencing. Four biological replicates were used for experimental data and evaluation.

2.9.2 Processing of RNA reads

Data from the sequencing were handled as specified in Kreszies *et al*. 2019. The raw sequencing data from IlluminaHiSeq 4000 sequencer (BGI Tech Solutions, Hong Kong, China)

consisted of 100 bp paired-end reads which were subsequently processed with CLC Genomics WORKBENCH v.10.0.1 (https://www.qiagenbioinformatics.com/). Reads that were >40 bp were mapped against the barley reference genome via ENSEMBLPLANTS:Hv_IBSC_PGSB_v2,v.2.36(ftp://ftp.ensemblgenomes.org/pub/plants/release-36/fasta/hordeum_vulgare/dna/) and the remaining reads with low-quality scores were excluded (Mascher *et al*., 2017). Unique reads with ≥ 80% of the length and ≥ 90% of identity to the reference were mapped. Stacked reads with indistinguishable 5' and 3' coordinates and orientation were consolidated and removed. The remaining reads were subjected to high confidence annotation with barley genome (Mascher *et al*., 2017), ftp://ftp.ensemblgenomes.org/pub/plants/release-36/fasta/hordeum_vulgare/dna/). The criteria for mapping to the high confidence gene set model includes that the sequence should be matched with ≥ 90% of the length and ≥ 90% of similarity. Reads with hit results of more than one were not considered and therefore eliminated from the counting. Normalization of the read counts by the sequencing depth and $\log_2$ transformation was done to fit the postulates of a linear model prior to the differential analysis. To compensate for the heteroscedasticity, precision weights were allocated for each variance points with the help of mean-variance estimation (Law *et al*., 2014).

2.9.3 Differential gene expression analysis

Multidimensional scaling plot (MDS) is one of the most significant exploratory plots to examine gene expression analysis (Smyth *et al*., 2018). The plot was developed using the plotMDS function implemented in the Bioconductor package LIMMA in R (R v.3.4.0, limma_3.32.2). The resulting two or three-dimensional plots reflect on the consistency of expression pattern within the group of biological replicates and also the separation of the groups of replicates that are compared (Ji and Sadreyev, 2018). Distance on the plot conforms to the leading fold change which is the average Log_2 FC (root mean square) for 500 genes that are most divergent between each pair of samples by default. An illustrated linear model to depict the differential gene expression between the control and salt-stressed roots was developed.

2.9.4 Statistical analysis of differentially expressed genes

Empirical Bayer moderation was performed to acquire the most precise estimates of gene variability (Smyth, 2004). Linear modelling in R package *Limma* was executed using contrast. fit function. The estimate of statistical significance of absolute differential expression was calculated as false discovery rate (FDR). For the sake of statistical significance, P values of the performed pairwise t-tests, FDR was adjusted ≤ 5% and this furthermore would be desirable for the definitive identification of differentially expressed genes.

2.9.5 Gene ontology enrichment analysis

Gene ontology is a term used to categorize differentially expressed gene products into molecular function, biological process and cellular component. The analysis was done via web-based agriGO v2.0 software (Su *et al*., 2018). Singular enrichment analysis (SEA) was performed by comparing the list of differentially expressed genes with the customized annotated reference from the IPK Barley BLAST server. The results were further combined and cross-compared with the SEACOMPARE tool in the agriGO v2.0 software.

2.10 Stomatal conductance measurement

The rate of stomatal transpiration was measured using an AP4 porometer (Delta-T Devices, England). Along with control, leaves stressed with 80 mM, 180 mM and 275 mM salt solution were subjected to the experiment. Readings were taken immediately after the implementation of stress and after 30 minutes from the well-developed leaves. Subsequent measurements were made once an hour for 5 hours for 3 days. Three biological replicates with 5-6 leaves per replicate per treatment were used for each day of the experiment.

2.11 Photosynthetic yield measurement

The impact of salinity on the photosynthetic yield was measured in the form of a light curve using a PAM fluorometer (Junior-PAM, Heinz Walz GmbH, Germany) with PAM (Pulse Amplitude Modulation) technique.

The readings were obtained by the incidence of actinic light on the leaves of both control and stressed, at a time interval of 5 min. The intensity of the light was varied progressively from 25 to 625 µmol.m².s^{-1}. The experiment was conducted for 3 days from the time of stress application on both Scarlett and wild barley from Pakistan. Data were obtained in the form of photosynthetic yield (of Photosystem II) and electron transport rate (ETR). Three biological replicates were used for each treatment.

2.12 Wax and cutin analysis

Wax and cutin analysis was done for control and salt-stressed 12 d old leaves (both Leaf 1 and Leaf 2) that had been treated with 180 mM NaCl which corresponds to the water potential of -0.8 MPa. The growth conditions were the same as that was described in section **1**.

2.12.1 Wax extraction and GC analysis

Wax extraction was done as described in Kurdyukov *et al*., 2006. 12d old barley leaves were detached from the plants and immediately dipped in 2 ml chloroform for 20 s at room temperature. Caution was taken to not damage the wax layer by touching the leaf surface. The area of the leaf was determined using Image J software (ImageJ.net) (Schneider *et al*., 2012). The solution with the wax content was spiked with 10 µg of tetracosane as an internal standard followed by evaporation with nitrogen gas. Derivatisation of the samples was done as elaborated in section **2.5.1.**

Derivatised samples were transferred into the auto sampler vials and further subjected to GC-MS/ FID analysis. The working principle was as described in section **2.5.2.** The temperature profile of GC-MS/ FID for wax analysis is as follows

Table 8 - Temperature profiles of GC MS and GC-FID for the analysis of wax

GC MS	Temperature rise (˚C/min)	Final temperature (˚C)	Temperature hold (min)
Wax		50	2
	40	200	2
	3	310	30

GC FID	Temperature rise (˚C/min)	Final temperature (˚C)	Temperature hold (min)
Wax		50	2
	40	200	2
	3	310	30

The results were obtained and interpreted as mentioned in section **2.5.2.**

2.12.2 Cutin extraction and GC analysis

The leaves (previously used for wax extraction) were immersed in $ChCl_3$: Ch_3OH (1:1) for 2 weeks at room temperature with constant shaking. The samples were dried on polytetrafluoroethylene (PTFE) in an activated silica gel desiccator and the dry weight was determined using an analytical balance (Sartorius CPA225D weighing balance) with a resolution of ± 0.01 mg. The samples were then trans-esterified (as explained in section **2.5.1**). 10 µg of internal standard C_{24} (dotriacontane) was added, followed by derivatisation and the extraction was performed with reference to the protocol explained in (section **2.5.2**). Three biological replicates were used for the analysis of each variety. The procedure for result acquisition and apprehension for GC-MS/FID was same as that for suberin and has been elaborated in section **2.5.2**. The temperature profile for cutin is as follows

Table 9 - Temperature profiles of GC MS and GC-FID for the analysis of cutin

GC MS	Temperature rise (˚C/min)	Final temperature (˚C)	Temperature hold (min)
Cutin		50	2
	45	200	1
	3	300	15
GC FID	**Temperature rise (˚C/min)**	**Final temperature (˚C)**	**Temperature hold (min)**
Cutin		50	2
	10	200	1
	3	310	20

2.13 Statistical analysis

The data acquired from the chemical analytics and physiological experiments were statistically investigated for three biological replicates for each experimental condition for both Scarlett and wild barley of Pakistan accession with the software, Origin Pro 9.0.0 (64-bit) SR2 b87. One-way analysis of variance (ANOVA) with Fisher's LSD test ($p<0.05$) was implemented to evaluate significant differences between means in the concentration among different experimental setups.

3 RESULTS

3.1 Shoot and root length measurements

The relative lengths of shoot and root in 12 d old barley seedlings were assessed in both control and stress environments. In the cultivar Scarlett, the shoot and root lengths decreased significantly with increasing salt concentrations. Compared to a shoot length of 23.6 ± 2 cm in control, there was a decrement observed in 80 mM (20.9 ± 3.7 cm), 180 mM (13.5 ± 1.6 cm) and 275 mM (11.8 ± 1 cm) (**Figure 3** A). The root lengths also decreased in accordance with stress intensity. From the control with an average of 24.1 ± 2.2 cm, there was a significant reduction in 80 mM, 180 mM and 275 mM treated roots with average lengths of 21.7 ± 3.1 cm, 16.7 ± 2.6 cm and 12.2 ± 1.4 cm respectively (**Figure 4** A).

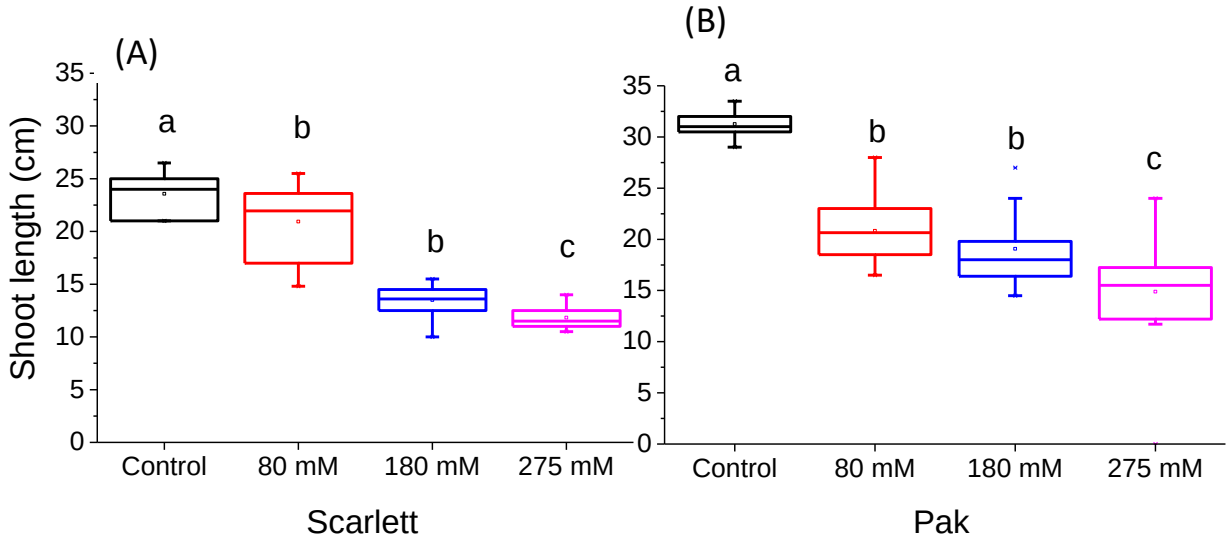

Figure 3 Shoot length of 12d old barley leaves

(A) Cultivar Scarlett and (B) Pakistan wild barley. The plants were grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. The boxes represent the 25th-75th percentile range and the square box inside represents the mean value. The whiskers indicate the minimum and maximum outliers range. Each box corresponds to data obtained from at least 30 barley shoots for each treatment and different alphabets denote significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

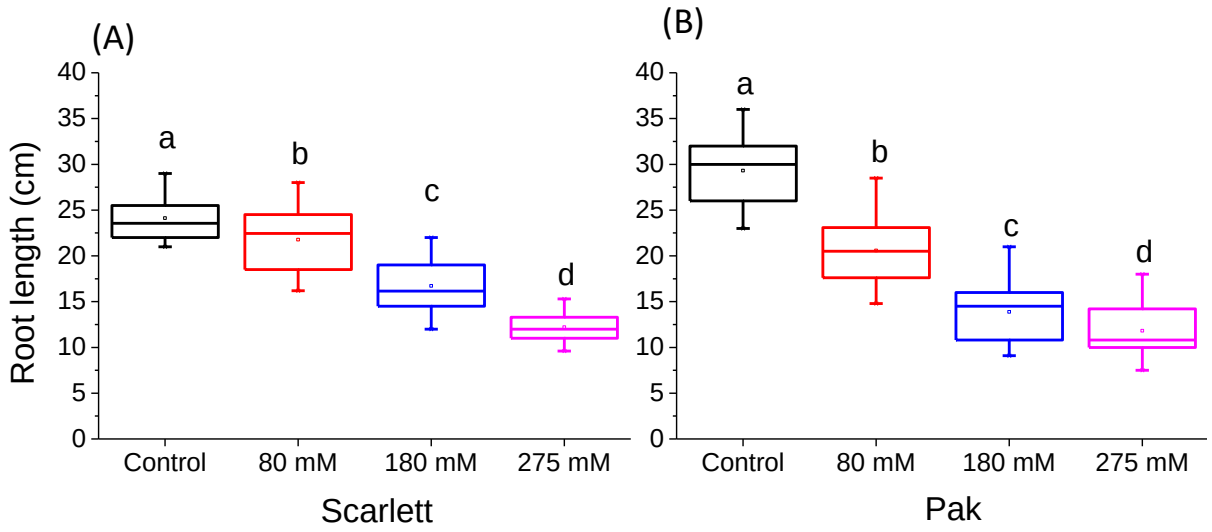

Figure 4 Root length of 12 d old barley seminal roots

(A) Cultivar Scarlett and (B) Pakistan wild barley. The plants were grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. The boxes represent the 25th-75th percentile range and the square box inside represents the mean value. The whiskers indicate the minimum and maximum outliers range. Each box corresponds to data obtained from at least 50 individual barley seminal roots for each treatment and different alphabets denote significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

The shoot and root lengths of the wild barley Pakistan also varied significantly under the implementation of salt stress. The shoot length from control (31.3 ± 1.3 cm) reduced to 20.8 ± 3.1 cm in 80 mM, 19.1 ± 3.9 cm in 180 mM and 14.9 ± 5.3 cm in 275 mM stressed leaves (**Figure 3** B). The root lengths also significantly decreased, the controls had an average of 29.3 ± 4.2 cm compared to the stressed measurements of 20.5 ± 3.3 cm, 13.8 ± 3.2 cm and 11.8 ± 3.1 cm under salt stress of concentrations 80 mM, 180 mM and 275 mM respectively (**Figure 4** B).

On comparing the relative growth measurements of controls between the cultivar Scarlett and the wild barley Pakistan, the latter had a longer shoot and root length. However, the % decrease was higher in wild barley Pakistan than in Scarlett. A maximum decline of 53 % in shoot length was observed in 275 mM stressed Pakistan wild barley compared to 50 % decrease in Scarlett. The root lengths decreased at a range of 30-59.7 % in Pakistan wild barley, whereas in Scarlett it was 9.9 - 49.37 % at different salt levels. Thus, wild barley from Pakistan showed greater decrease in shoot and root length compared to the Scarlett.

3.2 Histochemical assessment

The pattern of suberization in response to the salt stress across the different root lengths was observed through microscopic anatomization. **Figure 5** shows that the anatomy of a 12-day old seminal root consisting of one central and eight peripheral metaxylem vessels.

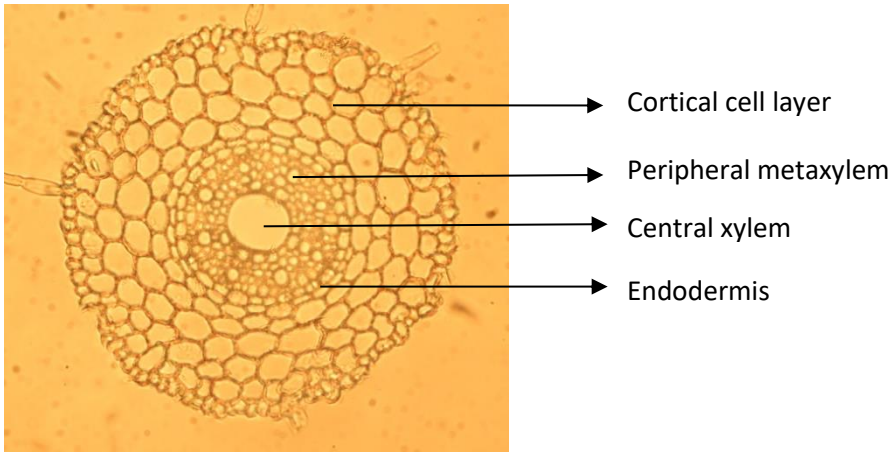

Figure 5 Cross-section of the 12d old barley seminal root of Scarlett

The root was grown under control conditions and viewed in bright field microscopy. The endodermis is surrounded by four cortical cell layers with one central and eight peripheral metaxylem vessels.

In order to determine the suberization degree along the root length, two types of staining were employed. The findings are as follows.

3.2.1 Microscopy of Scarlett roots

Flurol yellow 088 staining specifically illuminate the suberin lamellae which were seen as bright yellow depositions across the endodermal cells (**Figure 6**). In Scarlett, the extent of suberization was more prevalent in the stressed roots than in the control roots. Especially at the root tip which constitutes to 0-12.5 % of the total root length, complete suberization was achieved in contrast to that of control roots where little to no suberin lamellae was developed (**Figure 6** A-D). Moving forward, at 37.5 % of the length, a pattern of patchy suberin deposition was observed in control roots but complete suberization in the stressed roots grown in hydroponics with salt concentrations of 80 mM, 180 mM and 275 mM (**Figure 6** E-H). Towards the basal part of the roots (75-100% of total root length), there occurred complete development of suberin lamellae in both control and stressed roots

(**Figure 6** I-L). This pattern in the degree of suberization was persistent and it confirmed that the stressed had significant induction of suberin lamellae across the endodermis.

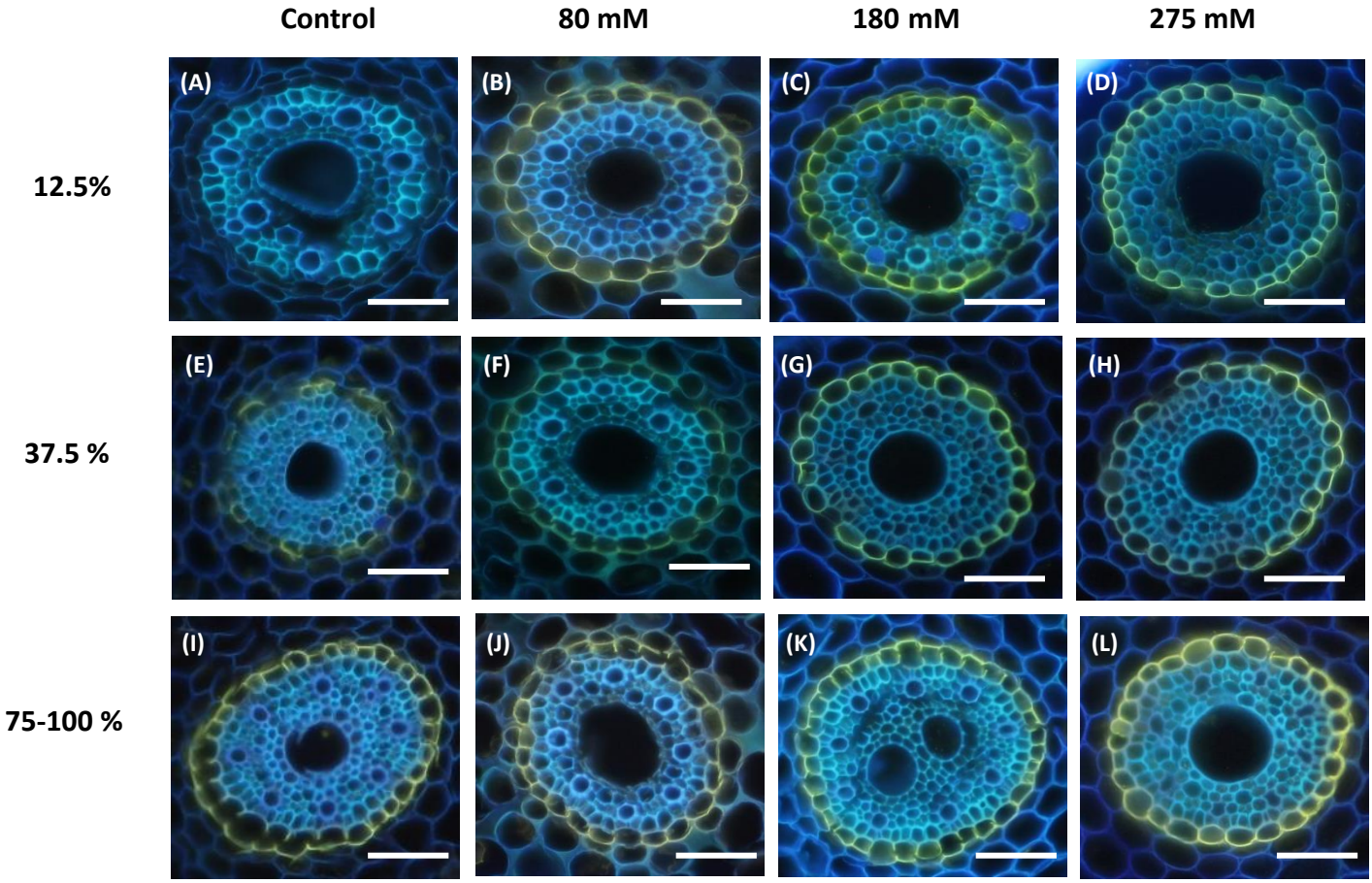

Figure 6 Flurol yellow 088 staining of endodermal suberin lamellae in 12 d old seminal roots of cultivated barley, Scarlett

The roots were grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Cross-sections were made at different lengths along the roots. (A) to (D) represents suberin lamellae development at 12.5 %; (E) to (F) at 37.5 % and (I) to (L) at 75-100 % of the root length. Scale bar represents 50 µm.

To visualize the presence of endodermal Casparian bands that were radially arranged across the cell walls, Berberine- aniline blue staining was used. In Scarlett, the development of Casparian bands had commenced at the root tip level (**Figure 7** B-D) with a greater number of suberized cells in stressed roots than in the control at least until 50% of the root length. The matured portion of the roots had Casparian bands developed completely in both control and salt-stressed roots (**Figure 7** I-L)

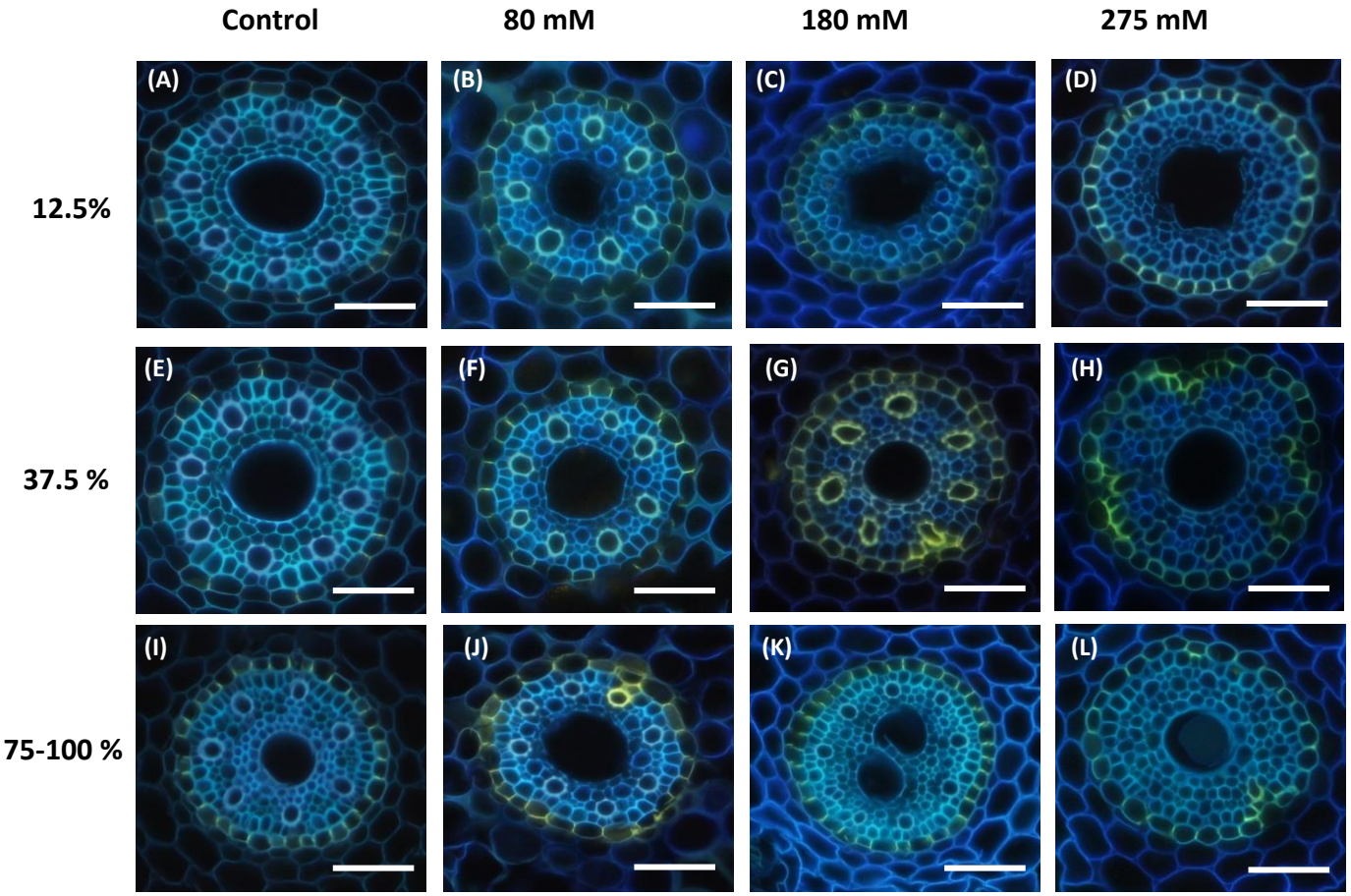

Figure 7 Berberine - Aniline blue staining of Casparian bands in 12 d old seminal roots of cultivated barley, Scarlett

The roots were grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Cross-sections were made at different lengths along the roots. (A) to (D) represents suberin lamellae development at 12.5 %; (E) to (F) at 37.5 % and (I) to (L) at 75-100 % of the root length. Scale bar represents 50 µm.

3.2.2 Microscopy of Pakistan wild barley roots

In wild barley Pakistan, similar to the cultivar Scarlett the stressed roots showed pronounced suberin depositions compared to the control especially at the younger part of the root which contributes to 0-12.5 % of entirety root length **(Figure 8** A, E). The roots at 37.5 % of length were 90 % suberized (**Figure 8** B, F) followed by complete suberin formation from 50-100 % of the root length in both control and stressed conditions (**Figure 8** C, D, G, H). Concerning the wild barley Pakistan, the crucial difference between the control and roots stressed with 180 mM salt was found at the developing root tips where the suberization was predominant in the latter than the control.

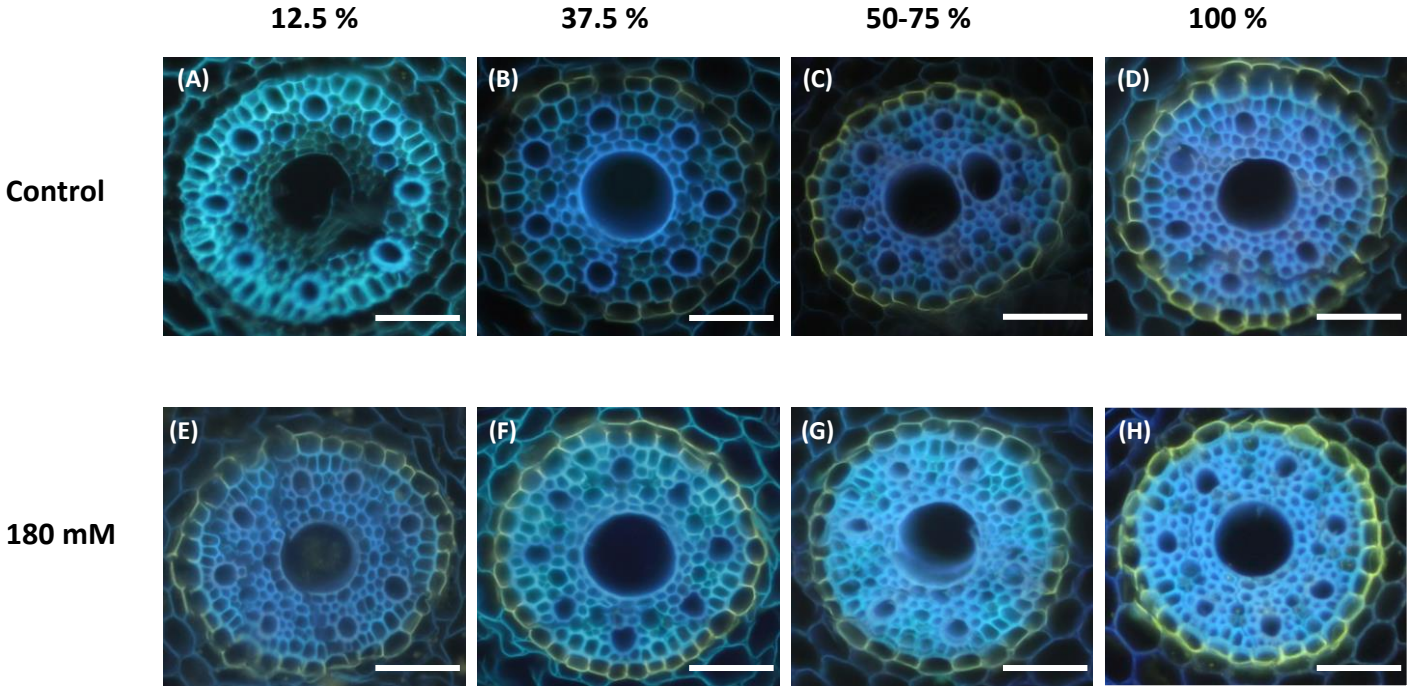

Figure 8 Flurol yellow 088 staining of endodermal suberin lamellae in 12 d old seminal roots of wild barley of Pakistan accession

The roots were grown in control and 180 mM salt stress concentration which corresponds to a water potential equivalent of -0.8 MPa. Cross-sections were made at different lengths along the roots. (A) and (E) represents suberin lamellae development at 12.5 %; (B) and (F) at 37.5 %; (C) and (G) at 50-75 %; (D) and (H) at 100 % of the root length. Scale bar represents 50 µm.

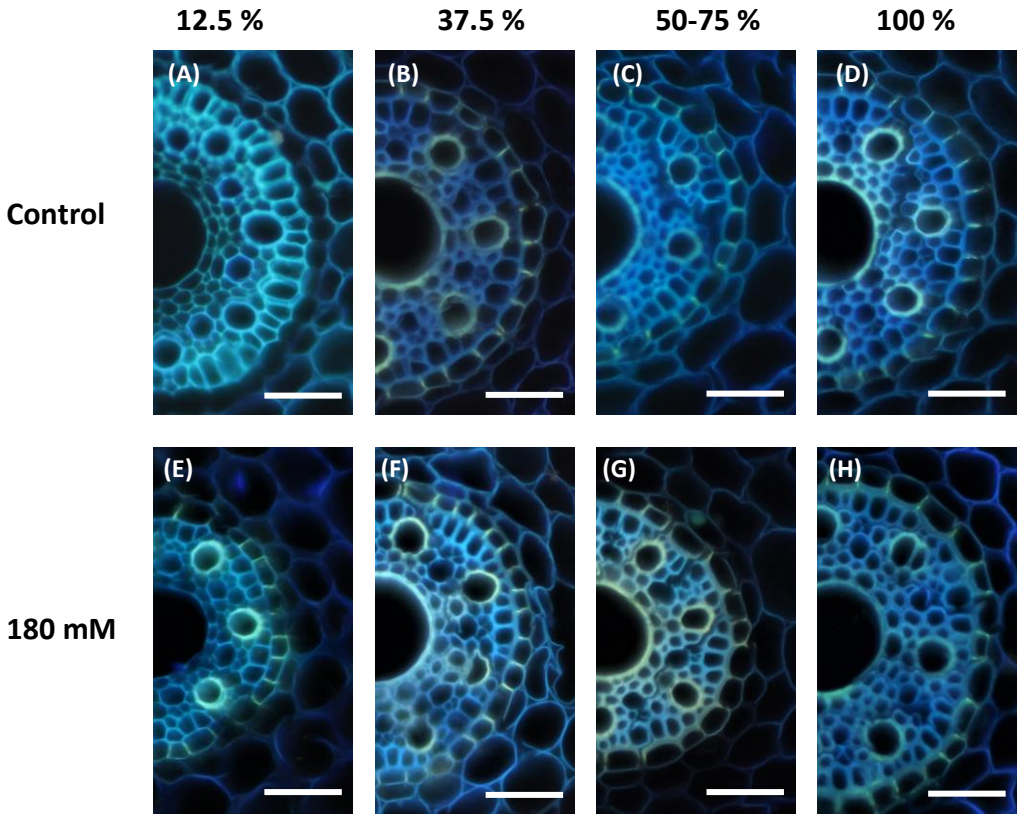

Figure 9 Berberine- aniline blue staining of Casparian bands in 12 d old seminal roots of wild barley of Pakistan accession.

The roots were grown in control and 180 mM salt stress concentration which corresponds to a water potential equivalent of -0.8 MPa. Cross-sections were made at different lengths along the roots. (A) and (E) represents suberin lamellae development at 12.5 %; (B) and (F) at 37.5 %; (C) and (G) at 50-75 %; (D) and (H) at 100 % of the root length. Scale bar represents 50 µm.

The Berberine- aniline blue staining in Pakistan wild barley (**Figure 9** A-H) showed a similar deposition trend as observed in the modern cultivar, Scarlett.

From the histochemical observation of suberin lamellae staining, the pattern of suberization at the level of 12.5 % of root length was similar in both modern cultivar Scarlett and wild barley from Pakistan. The number of suberized cells increases along the maturation of the cells over the root length.

3.3 Chemical analytics of suberin root content

For the chemical analysis of suberin content, based on the observations from the microscopic staining, the roots were categorized into different zones as described in Kreszies *et al*., 2019. In the cultivar Scarlett, the roots were divided into three zones – A, B and C with A constituting 0-25 %, B: 25-50% and C: 50-100 % of total root length respectively. Since wild barley had longer roots, the basal portion (50-100 %) was further divided into 50-75 % (zone C) and 75-100 % (zone D) of root lengths. For the GC analytics, ten root fragments from each zone were pooled together for a replicate. Likewise, three biological replicates were used for each zone per treatment. GC-MS/FID was performed as described in section **2.5**

Transesterification of the enzymatically treated 12d old barley seminal root segments resulted in the disintegration of suberin polymer into aromatic and aliphatic fractions. These constituents of suberin were identified and quantified by GC-MS/ FID. The concentration was calculated with relative to the area of the endodermis. The aromatic part mainly comprised of isomers of coumaric acid and ferulic acid. The aliphatic fraction included alcohols, fatty acids, diacids and ω-hydroxy acids with chain length varying from C_{16} to C_{26} amongst which $C_{18:1}$ diacid and C_{18} and C_{24} ω-OHs were found in abundance.

3.3.1 Suberin analytics in the roots of Scarlett

The total aliphatic content in the stressed seminal roots of Scarlett increased significantly along the lengths of the root compared to the control (**Figure 10**). The concentration in stressed roots of different salt concentrations ranged between 2.31 ± 1.27 and 3.86 ± 1.68 µg.cm^{-2} in Zone A with 4.1 fold increase in 80 mM; 5.3 fold increase in 180 mM and 6.8 fold increase in 275 mM NaCl treated roots. In Zone B, the total amounts varied significantly with 6.75 ±0.28 µg.cm^{-2} in 180 mM and 8.8 ± 1.46 µg.cm^{-2} in 275 mM NaCl stressed roots with a positive fold change of 4.1 and 5.4 respectively. The concentration in Zone C spanned between 4.81 ± 1.22 and 9.07 ± 0.69 µg.cm^{-2} in mildly stressed 80 mM and strongly stressed 275 mM salt-treated roots respectively. In Zone A, there was no significant difference within the stress treatments; but in Zone B and C, the total aliphatics in 180 mM and 275 mM salt-treated roots varied significantly than the roots grown in 80 mM NaCl.

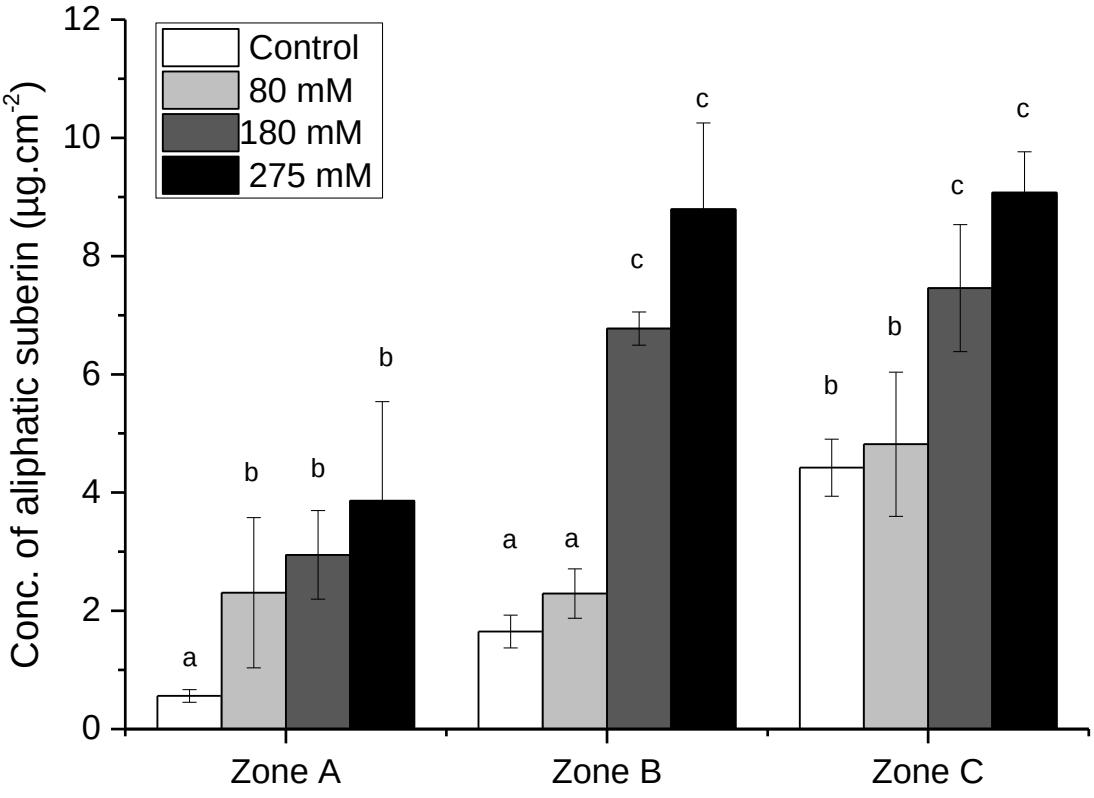

Figure 10 Total aliphatic suberin concentration in different zones of barley cultivar, Scarlett

The data corresponds to the concentration of total aliphatic suberin present in 12d old seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger

apical tip of the root (0-25 %); Zone B (25-50%) and Zone C, matured basal portion of the root (50-100%). The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 level in one way ANOVA (Fisher's least significant difference, LSD).

In terms of total aromatics, there was a significant change between the control and stressed roots (**Figure 11**). The values in Zone A ranged between 2.91 ± 1.1 and 2.72 ± 0.44 µg.cm^{-2} with a fold change of 2.3, 2.4 and 2.2 times in 80 mM, 180 mM and 275 mM salt concentrations respectively. Zone B amounted to the range 3.01 ± 0.27 and 5.73 ± 0.87 µg.cm^{-2} whereas in Zone C, it was between 8.37 ± 1.2 and 13.09 ±1.81 µg.cm^{-2}. In both Zone B and C, there was a significant increase in 180 mM and 275 mM salt-treated roots than in control. In Zone A, there was no significant difference within the stress treatments; but in Zone B and C, the total aromatics in 180 mM and 275 mM salt-treated roots varied significantly than the roots grown in 80 mM NaCl.

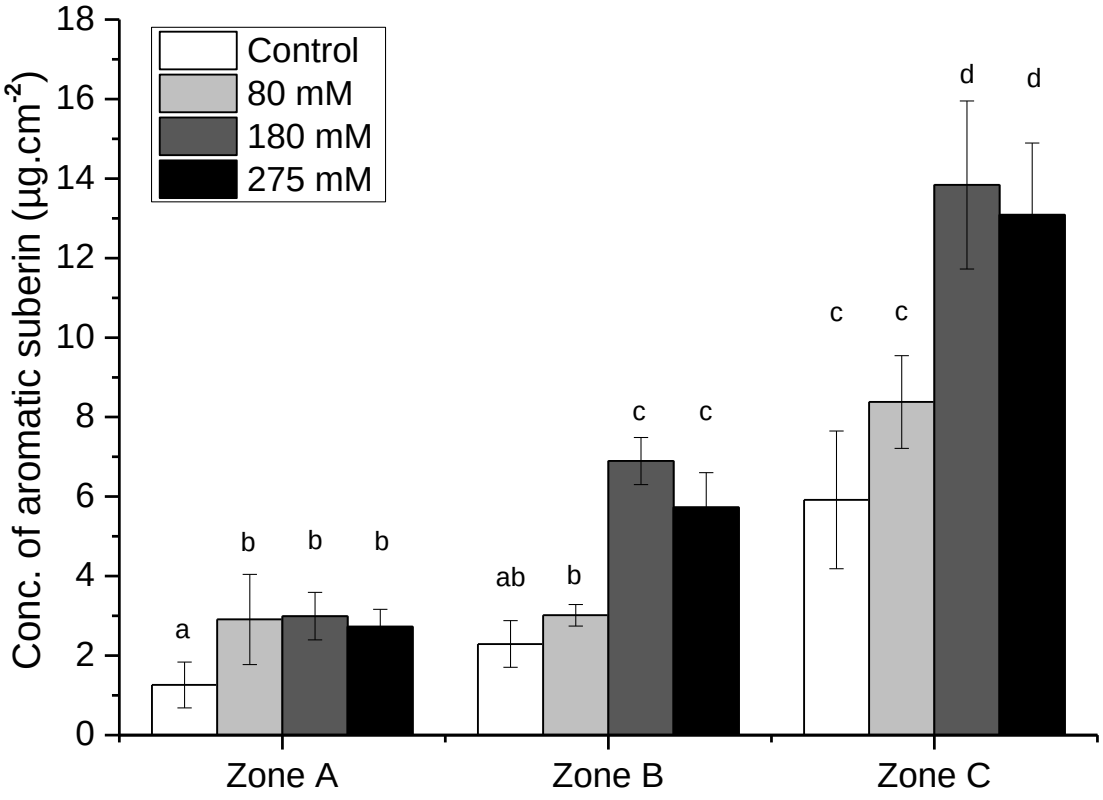

Figure 11 Total aromatic suberin concentration in different zones of barley cultivar, Scarlett.

The data corresponds to the concentration of total aromatic suberin present in 12d old seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger apical tip of the root (0-25 %); Zone B (25-50%) and Zone C, matured basal portion of the root (50-100%). The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes

significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

The aliphatic unit comprised of monomer classes including alcohols, diacids, fatty acids and ω-hydroxy acids. The concentration of the aliphatic classes increased with root length and was also higher in the salt-treated samples. ω-hydroxy acids contribute as the highest constituent followed by diacids (**Figure 12**). The concentration of ω-hydroxy acids with a maximum of 2.47 ± 1.12, 5.89 ± 1.5 and 5.9 ± 0.56 µg.cm^{-2} was found in Zone A, B and C respectively from the roots fragments of 275 mM salt treatment. There was a significant difference not only between the control and stressed but also between mild stress (80 mM) and stronger stress concentrations (180 mM and 275 mM). Diacids increased up to 1.4 ± 0.4 µg.cm^{-2} and 1.66 ± 0.13 µg.cm^{-2} significantly in Zone C of 180 mM and 275 mM treated roots respectively. The aggregates of alcohols and fatty acids in Zone C were almost twice the amount in Zone A.

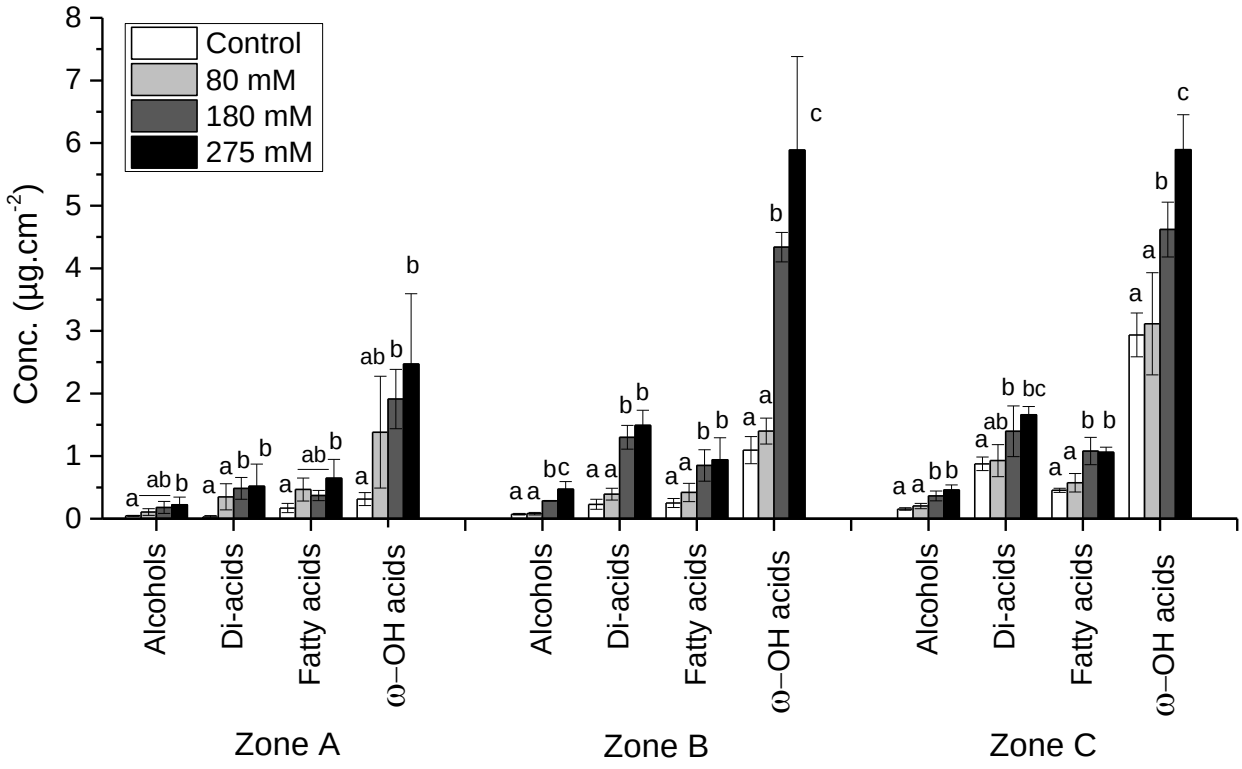

Figure 12 Aliphatic substance classes concentration in different zones of barley cultivar, Scarlett

The data corresponds to the concentration of aliphatic substance classes of suberin present in 12d old seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger apical tip of the root (0-25 %); Zone B (25-50%) and Zone C, matured basal portion of

the root (50-100%). The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

The individual monomeric units with the entire aliphatic composition have been shown in **Figure 13**. Throughout the monomers, significant differences were found particularly in stronger stress concentrations of 180 mM and 275 mM NaCl. The most profoundly found component was ω-hydroxy acids, followed by diacids, fatty acids and alcohols in that order. In Zone C, C_{18} and C_{24} ω-OHs contributed a maximum amount of 1.82 ± 0.09 µg.cm^{-2} and 1.58 ± 0.12 µg.cm^{-2} respectively at the highest concentration of 275 mM NaCl. C_{18} of diacids and fatty acids; and C_{16} alcohols were found in higher proportions with a maximum fold increase of 55.3 ($C_{18:1}$ diacids), 2.62 (C_{18} fatty acid) and 23 (C_{16} alcohol) in Zone C of 275 mM NaCl stressed plants.

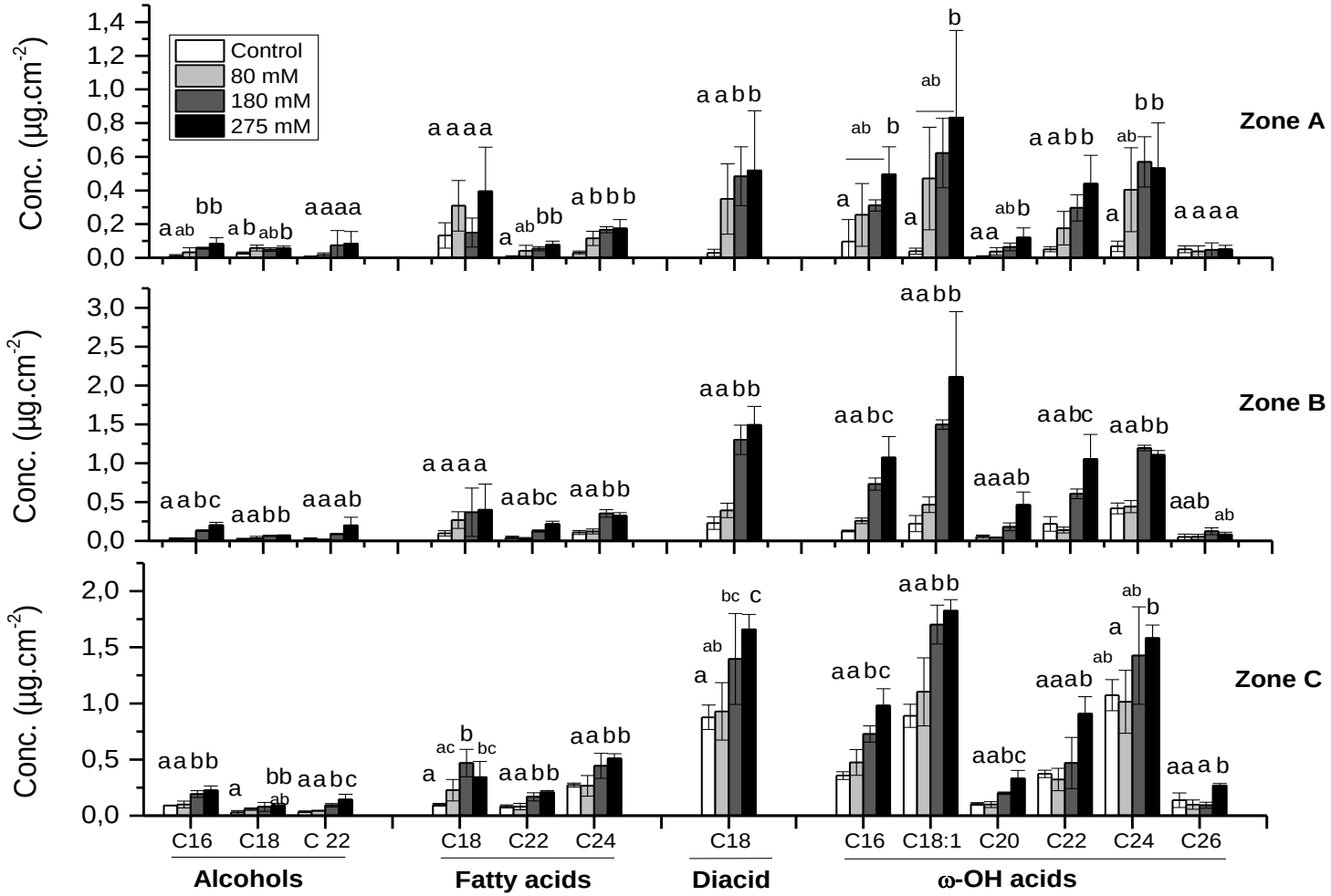

Figure 13 Aliphatic monomers concentration in different zones of barley cultivar, Scarlett

The data corresponds to the concentration of aliphatic monomers of suberin present in 12d old seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger apical

tip of the root (0-25 %); Zone B (25-50%) and Zone C, matured basal portion of the root (50-100%). The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

The relative amounts of aromatic monomers were shown in **Figure 14**. Similar to the aliphatic counterpart, the aromatic constituents also increased with stress concentrations along the root lengths spanning from Zone A to C. Comparably stronger induction was found in stresses infused with 180 mM and 275 mM NaCl which reflected on the significance increment across all the zones. The *trans* isomer of coumaric and ferulic acids were found in abundance than the *cis* isomers of the same. The amount of trans coumaric and ferulic significantly varied between 5.52 ± 1.29 $\mu g.cm^{-2}$ and 6.82 ± 2.38 $\mu g.cm^{-2}$; and 4.76 ± 1.92 $\mu g.cm^{-2}$ and 6.88 ± 0.43 $\mu g.cm^{-2}$ in Zone C of 180 mM and 275 mM respectively. Here also, like in aliphatics, there was a significant difference in amounts between the treatments; especially between 80 mM and stronger stress concentrations of 180 mM and 275 mM NaCl.

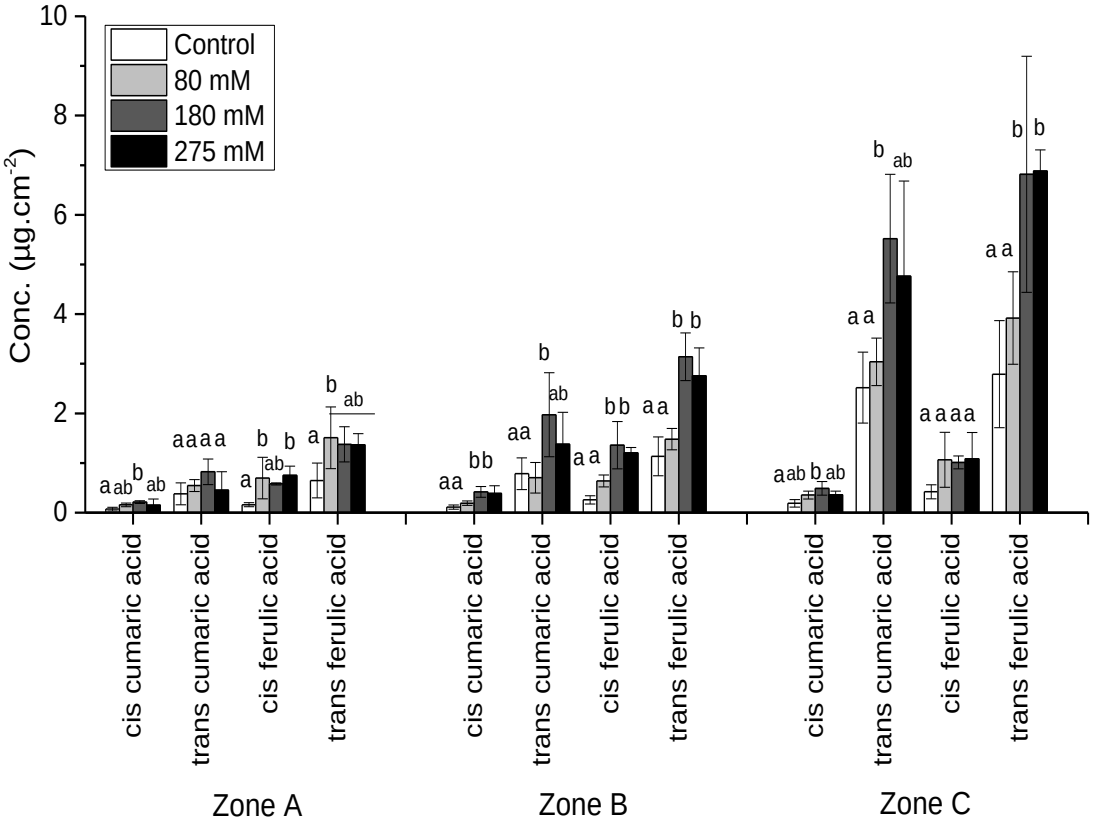

Figure 14 Aromatic monomer concentration in different zones of barley cultivar, Scarlett

The data corresponds to the concentration of aromatic classes of suberin present in 12d old seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger apical tip of the root (0-25 %); Zone B (25-50%) and Zone C, matured basal portion of the root (50-100%). The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

3.3.2 Suberin concentration in roots of wild barley of Pakistan accession

The graph depicting the total aliphatic suberin content is shown in **Figure 15**. There was a significant variance in the deposition of suberin between the control and stress treatments, across different root zones. There was a fold increase of 3.5, 4.3 and 5.1 in Zone A of the roots stressed with 80 mM, 180 mM and 275 mM respectively. The amount in the stressed samples ranged between 3.38 ± 1.12 µg.cm^{-2} and 4.95 ± 1.6 µg.cm^{-2} in Zone A; 3.24 ± 1.01 µg.cm^{-2} to 6.1 ± 0.71 µg.cm^{-2} in Zone B; 3.96 ± 0.42 µg.cm^{-2} to 6.06 ± 0.15 µg.cm^{-2} in Zone C and 5.80 ± 0.4 µg.cm^{-2} to 6.94 ± 0.39 µg.cm^{-2} in Zone D. From these values it was evident that the deposition of total aliphatic suberin sharply increased in the younger part of the roots and subsequently, the amounts became stagnant onward and upward until the matured basal portion of the roots.

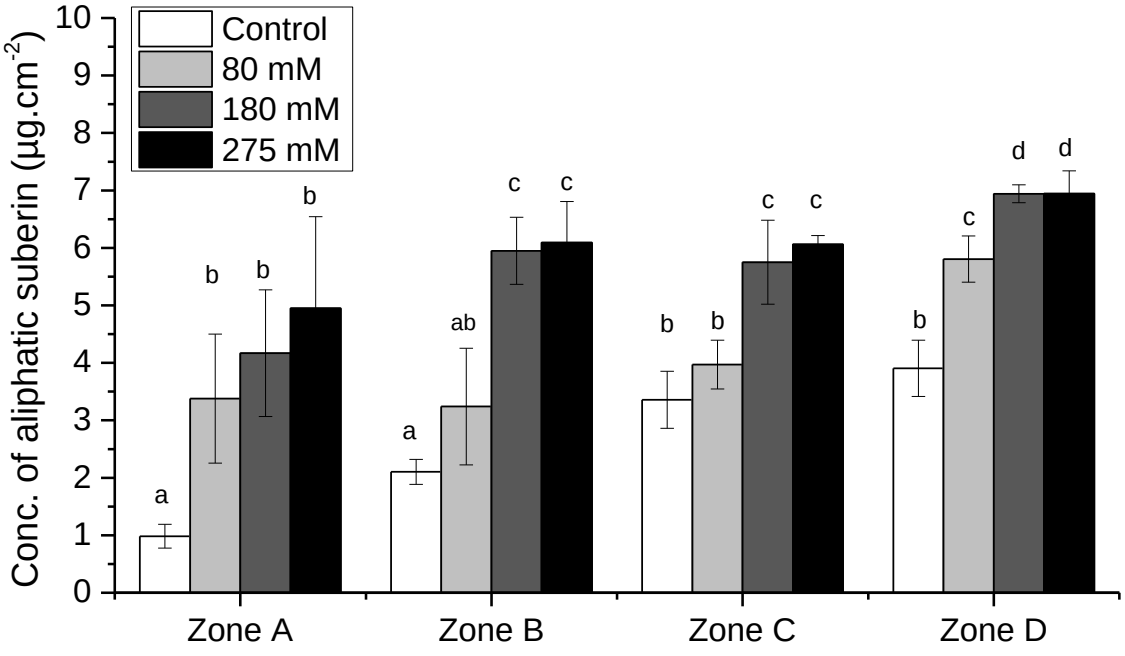

Figure 15 Total aliphatic suberin concentration in different zones of wild barley from Pakistan

The data corresponds to the concentration of total aliphatic suberin present in 12d old seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger apical tip of the root (0-25 %); Zone B (25-50%); matured basal portion of the root Zone C (50-75 %) and Zone D (75-100 %). The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

The total aromatic concentration (**Figure 16**) did not differ significantly between the control and stress conditions in Zones A and B. However in Zone C, there was a statistically significant difference between the control and roots stressed with stronger concentrations of 180 mM and 275 mM NaCl. The amounts were 11.22 ± 4.11 µg.cm^{-2} and 9.56 ± 4.4 µg.cm^{-2} in Zone C of 180 mM and 275 mM NaCl treated wild barley seminal roots.

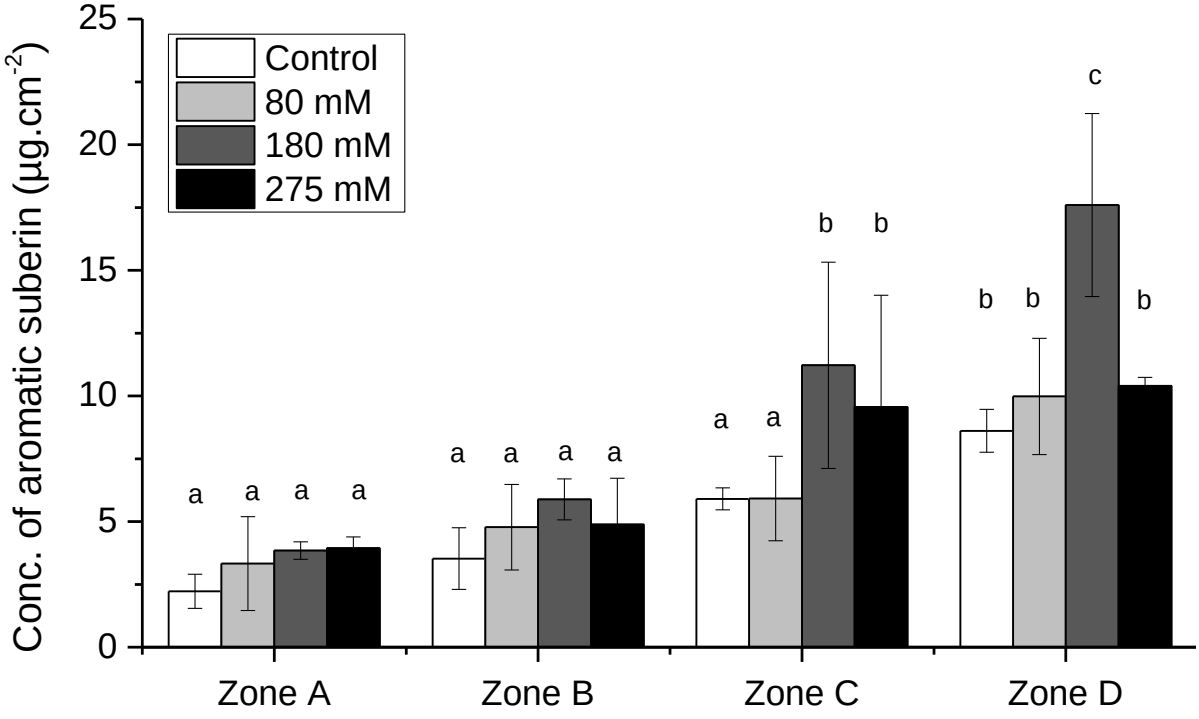

Figure 16 Total aromatic suberin concentration in different zones of wild barley from Pakistan

The data corresponds to the concentration of total aromatic suberin present in 12d old seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger apical tip of the root (0-25 %); Zone B (25-50%); matured basal portion of the root Zone C (50-75 %) and Zone D (75-100 %). The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

The aliphatic class monomers found in wild barley of Pakistan accession was analogous to that in modern cultivar Scarlett. The major constituents of the aliphatic unit in suberin polymer included alcohols, fatty acids, di-acids and ω-hydroxy acids. From **Figure 17**, it was affirmative that ω-hydroxy acids contributed the highest followed by di-acids. While there was significance in the amounts between the control and stressed plants, there was also an effect because of the intensity of the salt concentration applied. This maybe the reason why the mildly stressed roots with 80 mM NaCl differed significantly with stronger salt concentrations of 180 mM and 275mM. The aggregates of alcohols in stressed roots ranged between 0.09 ± 0.05 $\mu g.cm^{-2}$ and 0.34 ± 0.04 $\mu g.cm^{-2}$; fatty acids between 0.77 ± 0.16 and 0.99 ± 0.4 $\mu g.cm^{-2}$; di-acids from 0.71 ±0.56 $\mu g.cm^{-2}$ to 1.16 ± 0.21 $\mu g.cm^{-2}$ and ω-hydroxy acids within 1.8 ± 0.53 $\mu g.cm^{-2}$ to 4.5 ± 0.46 $\mu g.cm^{-2}$. Up to 2-fold increase in ω-hydroxy acids was a major element in boosting the aliphatic suberin concentration in the stressed roots.

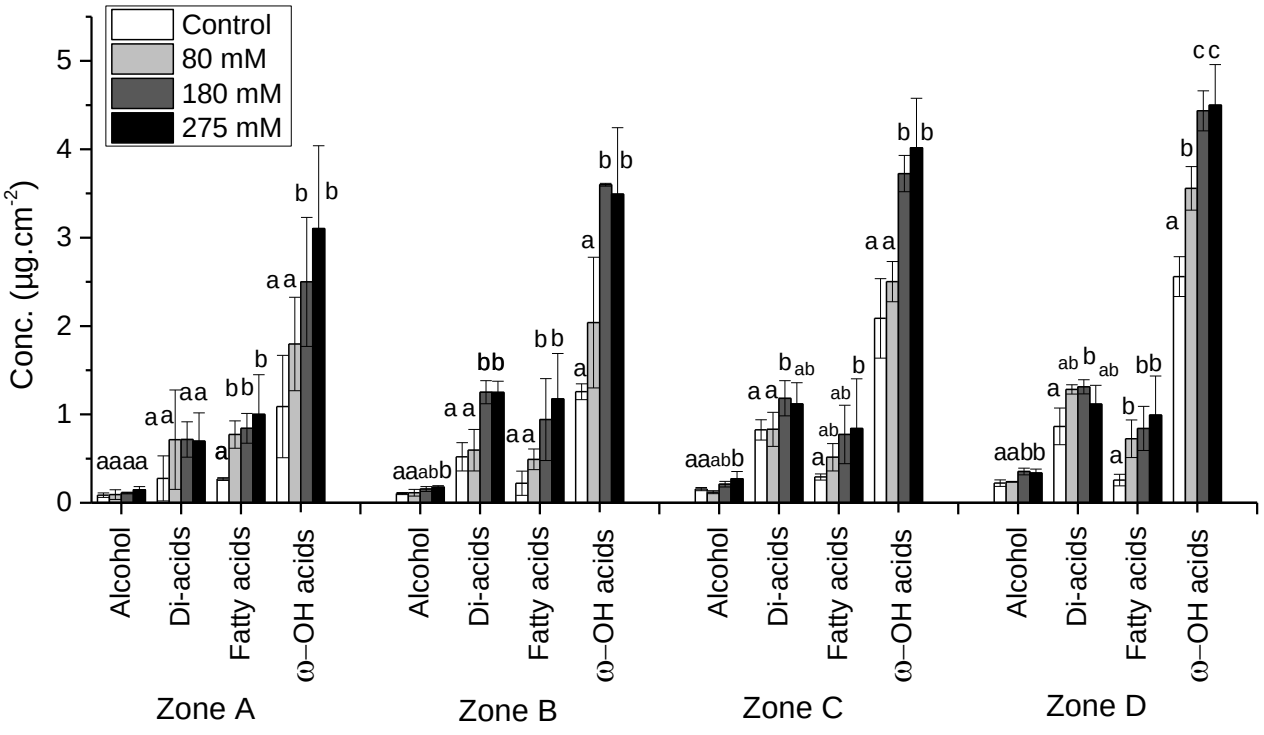

Figure 17 Aliphatic substance classes concentration in different zones of wild barley from Pakistan

The data corresponds to the concentration of aliphatic substance classes present in 12d old seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger apical tip of the root (0-25 %); Zone B (25-50%); matured basal portion of the root Zone C (50-75 %) and Zone D (75-100 %). The data represent the mean and standard deviation from three biological

replicates; Different alphabet denotes significant differences between the means at 0.05 level in one way ANOVA (Fisher's least significant difference, LSD).

The individual class monomers of aliphatics and aromatics were shown in **Figure 18** and **Figure 19** respectively. From the graph, it can be comprehended that C_{18} and C_{24} ω-OHs were found in abundance followed by $C_{18:1}$ di-acid. The concentration of C_{18} ω-OH ranged from 0.54 ± 0.3 $\mu g.cm^{-2}$ to 1.34 ± 0.07 $\mu g.cm^{-2}$; C_{24} ω-OH between 0.54 ± 0.22 $\mu g.cm^{-2}$ and 1.38 ± 0.2 $\mu g.cm^{-2}$; and $C_{18:1}$ di-acid from 0.66 ±0.54 $\mu g.cm^{-2}$ to 1.02 ± 0.14 $\mu g.cm^{-2}$ in stressed roots across all the root zones. Amongst the fatty acid concentrates, C_{24} was profoundly present with an average amount of 0.62 ± 0.2 $\mu g.cm^{-2}$ in stressed samples along the length of the roots. Lastly, in alcohols C_{16} monomer was predominantly found with up to 5 fold increase compared to the control.

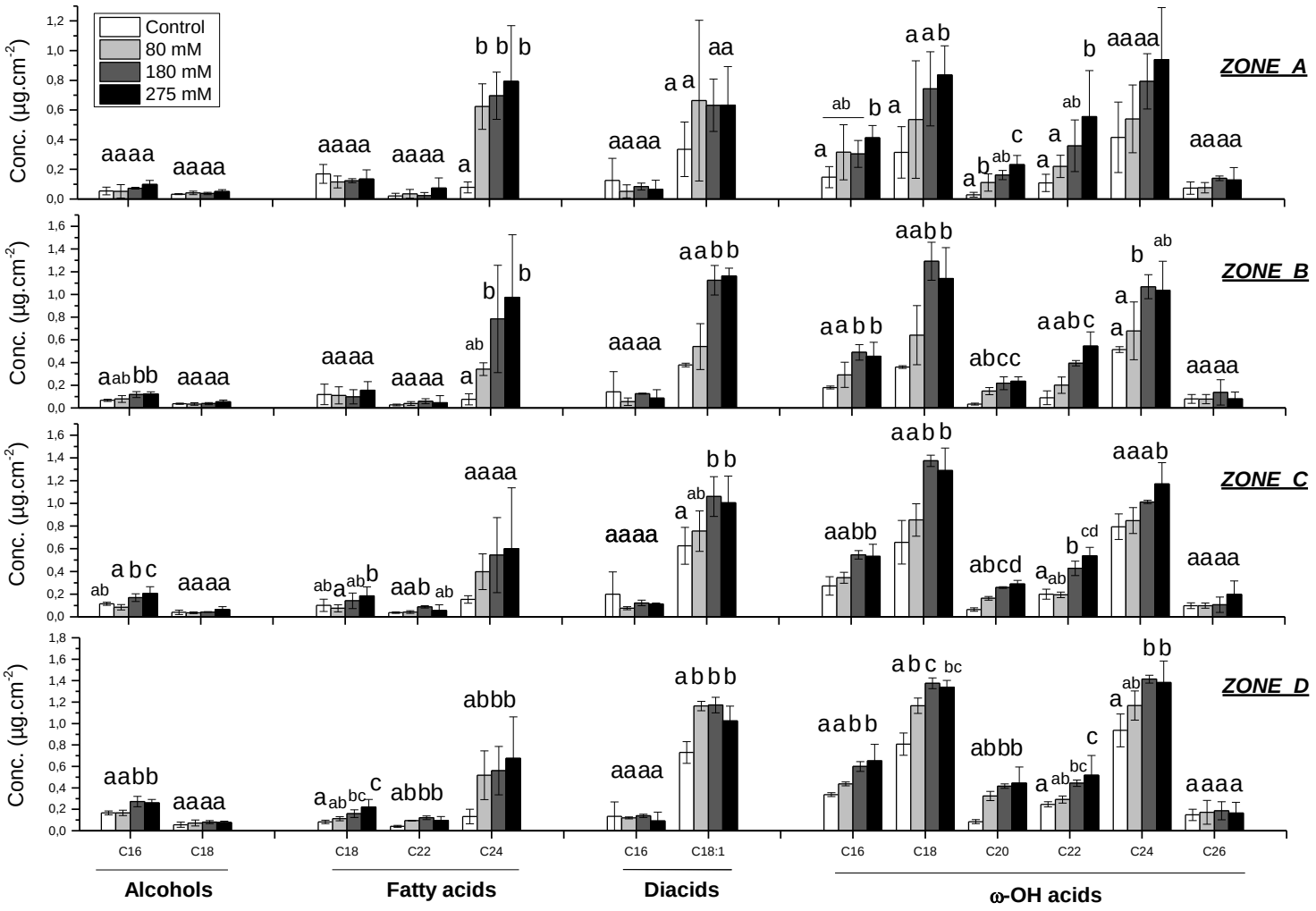

Figure 18 Aliphatic monomers concentration in different zones of wild barley from Pakistan

The data corresponds to the concentration of aliphatic monomers present in 12d old seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger apical tip of the root (0-25 %); Zone B (25-50%); matured basal portion of the root Zone C (50-75 %) and Zone D (75-100 %). The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

Monomers of aromatic suberin counterparts were shown in **Figure 19**. Among the isomers of coumaric and ferulic acids detected in chromatograms, *trans*-isomer aggregates were prevalently found than the *cis* forms. Significant differences in the quantity were seen in samples grown in higher salt concentrations of 180 mM and 275 mM NaCl. There was a maximum increment of 8.4 and 10.1 times fold change in *trans* coumaric acids and *trans* ferulic acids in Zone D of 275 mM salt-stressed roots.

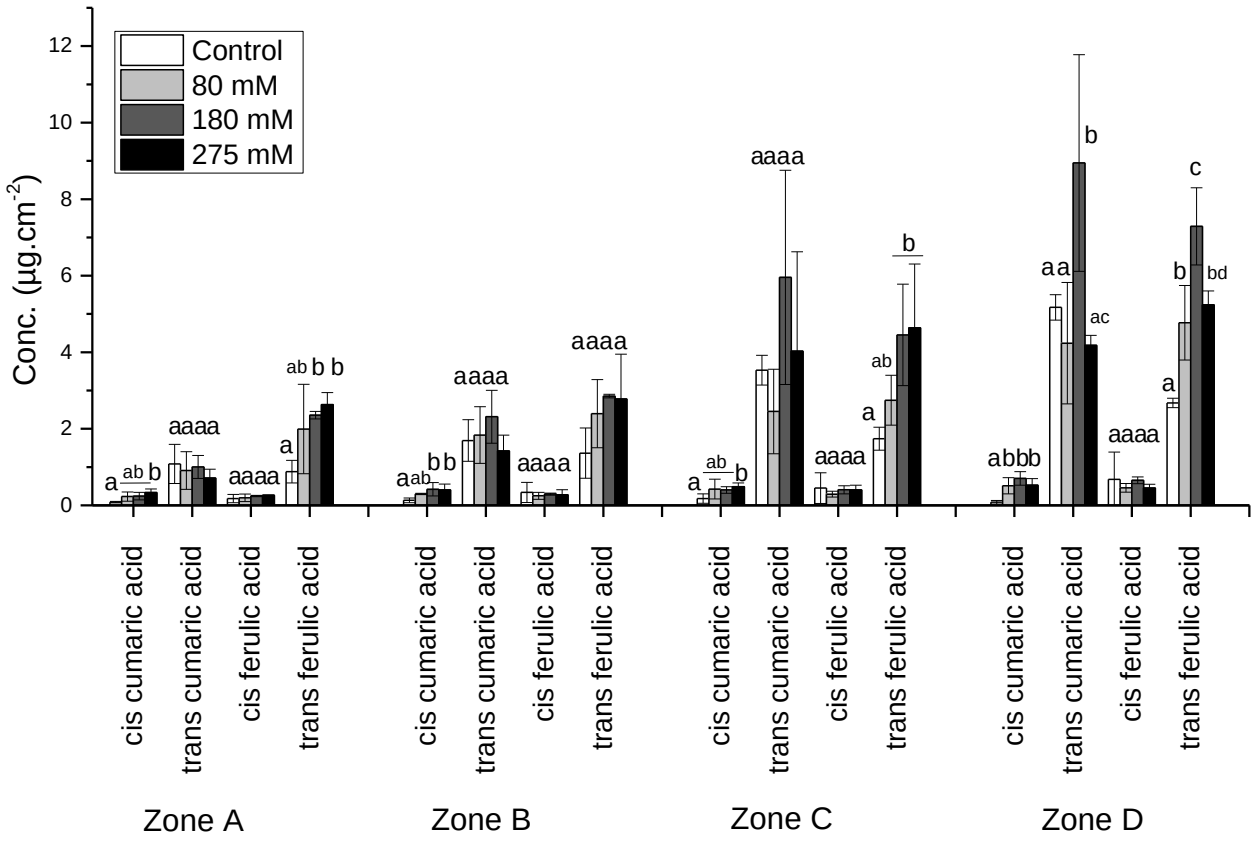

Figure 19 Aromatic monomers concentration in different zones of wild barley from Pakistan

The data corresponds to the concentration of aromatic monomers present in 12d old seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger apical tip of the root (0-25 %); Zone B (25-50%); matured basal portion of the root Zone C (50-75 %) and Zone D (75-100 %). The data represent the mean and standard deviation from three biological replicates; Different

alphabet denotes significant differences between the means at 0.05 level in one way ANOVA (Fisher's least significant difference, LSD).

3.3.3 Scarlett vs. wild barley from Pakistan

The total suberin amounts of aliphatics and aromatics unit in cultivar Scarlett and wild barley from Pakistan were compared in **Figure 20**. The pattern of suberization along the lengths of the root has been correlated. Previously in Pakistan wild barley, 50-100 % of the roots had been categorized into two zones – Zone C (50-75 %) and Zone D (75-100 %); however, for the convenience of comparison with Scarlett, 50-100 % part of the root was treated as a single zone (Zone C). From the graph, it was indicative that the amount of suberin deposition increases with stress concentrations along the root lengths. Also, there was a significant difference in the suberization norm between the control and stressed roots.

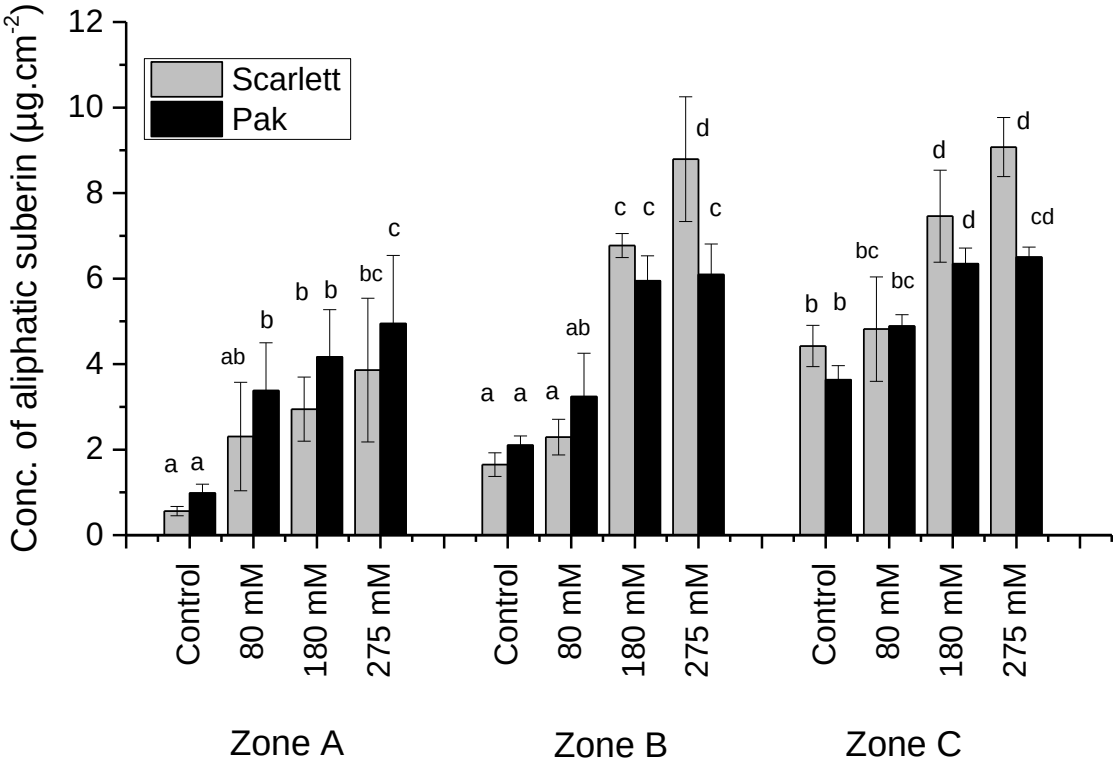

Figure 20 Total aliphatic suberin concentration in different zones of modern cultivar Scarlett and wild barley from Pakistan

The data corresponds to the concentration of total aliphatic suberin present in 12d old seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger apical tip of the root (0-25 %); Zone B (25-50%) and Zone C, matured basal portion of the root (50-100%). The

data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

Zone A which comprises 0-25 % of total root length, there was a stronger response in the initiation of suberization in Pakistan wild barley than in Scarlett. There was a maximum quantity of 3.86 ± 1.68 µg.cm^{-2} and 4.95 ± 1.59 µg.cm^{-2} in Scarlett and wild barley Pakistan respectively from the highest stress concentration of 275 mM NaCl. In the event of stronger stress induction by 180 mM and 275 mM NaCl, there was a shift in the suberization acceleration from Pakistan wild barley to Scarlett in Zone B i.e., 25-50 % of root length. A total of 8.79 ± 1.46 µg.cm^{-2} and 6.09 ± 0.71 µg.cm^{-2} was found in Scarlett and wild barley of Pakistan accession respectively. Zone C which contributed to 50 -100 % of the root length showed a substantial increment of suberin accumulation in the stressed roots of Scarlett than in Pakistan wild barley. Here a sum of 9.07 ± 0.69 µg.cm^{-2} and 6.5 ± 0.24 µg.cm^{-2} was found in cultivar Scarlett and wild barley of Pakistan accession.

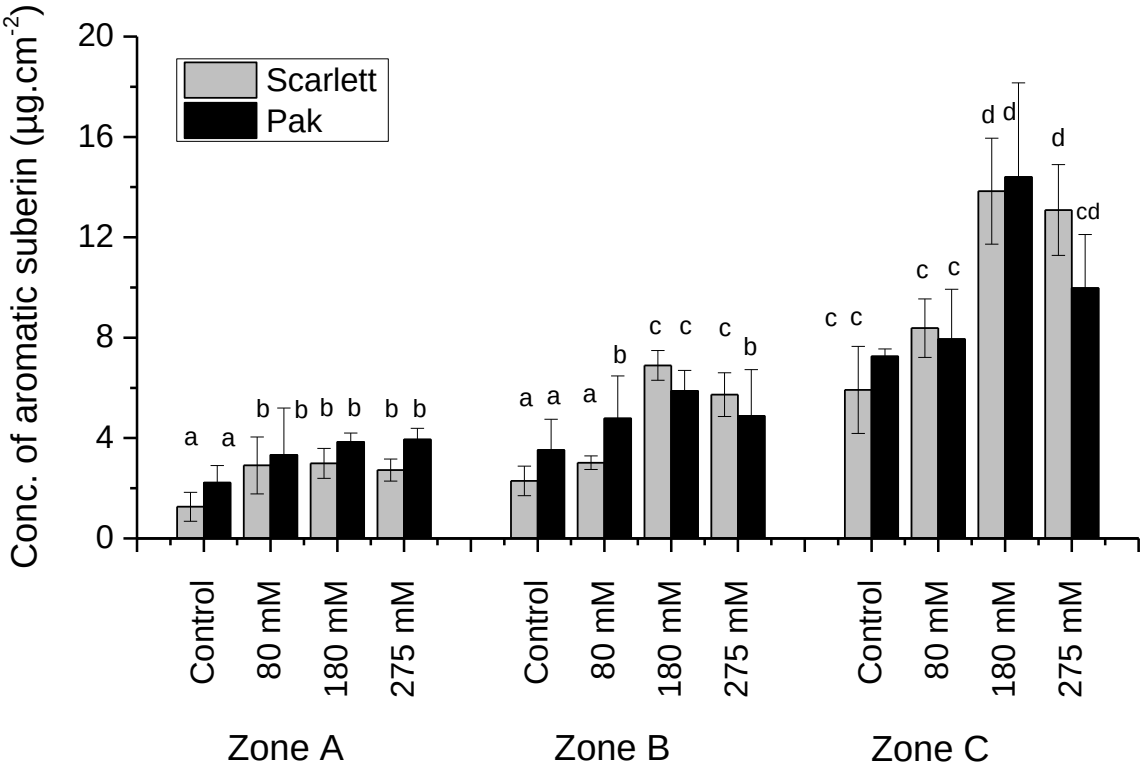

Figure 21 Total aromatic suberin concentration in different zones of cultivar Scarlett and wild barley from Pakistan

The data corresponds to the concentration of total aromatic suberin present in 12d old seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a

water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger apical tip of the root (0-25 %); Zone B (25-50%) and Zone C, matured basal portion of the root (50-100%). The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

The amounts of total aromatics (**Figure 21**) increased sharply over the length of the roots although no significant difference was found between the two genotypes in each treatment. Maximal of 13.09 ± 1.81 $\mu g.cm^{-2}$ and 14.41 ± 3.76 $\mu g.cm^{-2}$ was obtained in the stressed roots of Scarlett and Pakistan wild barley respectively.

3.4 ICP elemental variation analysis

To determine the concentration of Na ions present in the leaves and roots and also to determine the amounts of essential macro and micro elements within the tissue under the influence of salinity (80 mM), ICP analysis was done. Results were as follows. The micro nutrients such as copper (Cu), manganese (Mn) and zinc (Zn) did not vary in the leaves (**Figure 22** A, B**)** and roots (**Figure 23** A, B**)** of both barley varieties. The concentration of iron (Fe) differed significantly in stressed samples except in Scarlett leaves.

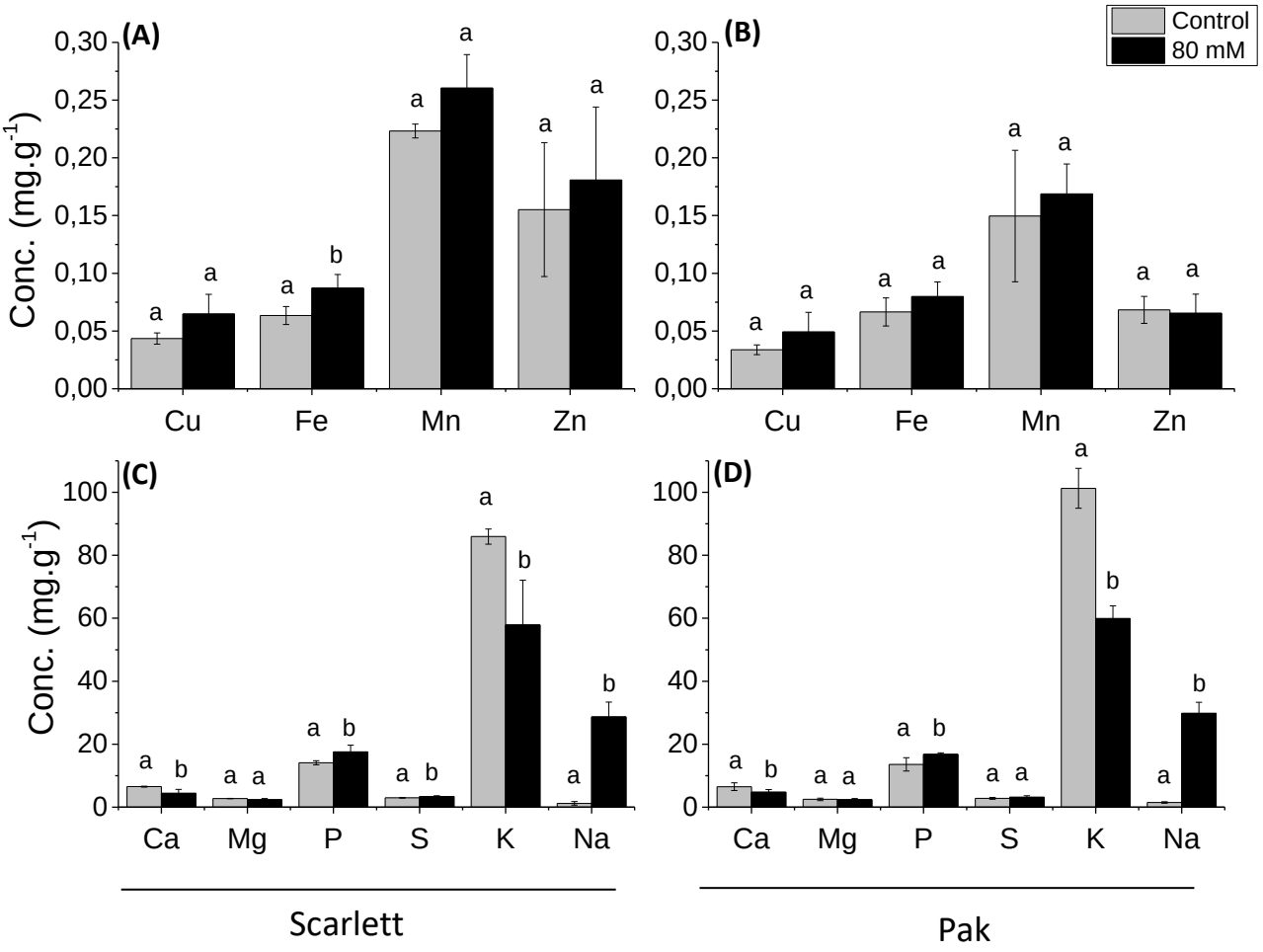

Figure 22 Determination of Na concentration and elemental variation under salt stress in the leaves of Scarlett and Pakistan wild accession

The plants were grown in control and salt stress concentration of 80 mM NaCl. The micro elements are shown in (A) and (B); macro elements along with Na are shown in (C) and (D). The data represent the mean and standard deviation from at least three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

In case of macro nutrients, calcium (Ca) and potassium (K) decreased significantly in the leaves (**Figure 22** C,D) and as well as in roots (**Figure 23** C, D) in both Scarlett and wild barley from Pakistan. Na accumulation was profoundly found in stressed leaves and roots. The amount of Na in Scarlett increased from 1.2 ± 0.5 $mg.g^{-1}$ in control to 28.65 ± 4.7 $mg.g^{-1}$ in stressed leaves and 3.8 ±0.6 $mg.g^{-1}$ in control to 27.9 ± 4.19 $mg.g^{-1}$ in stressed roots. In Pakistan also 20.1 and 12.1 fold increase was found in leaves and roots respectively. Thus, the increment in Na accumulation was subsequently accompanied by decrease in K and Ca levels in the stressed samples of both genotypes.

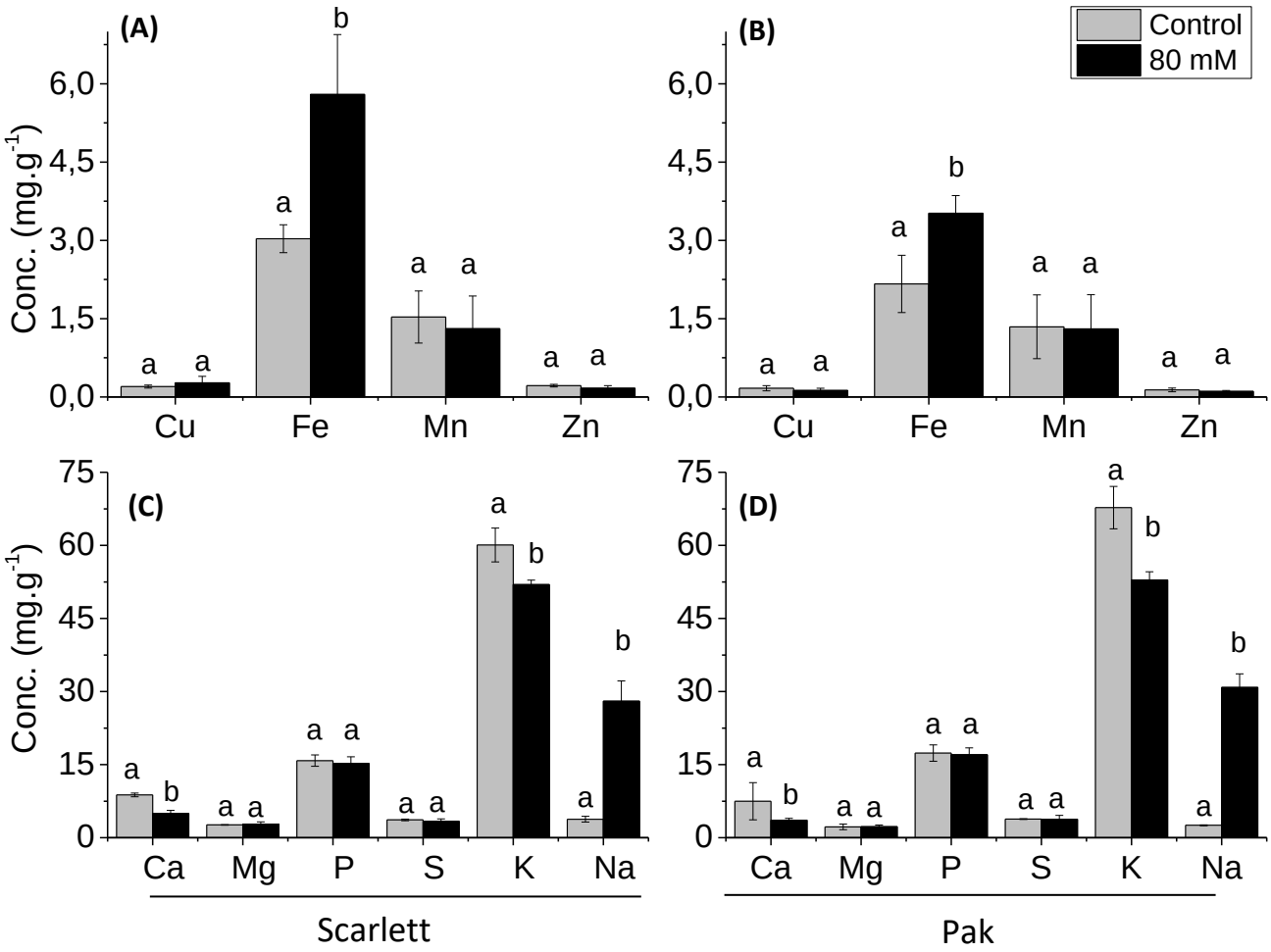

Figure 23 Determination of Na concentration and elemental variation under salt stress in the roots of Scarlett and Pakistan wild accession

The plants were grown in control and salt stress concentration of 80 mM NaCl. The micro elements are shown in (A) and (B); macro elements along with Na are shown in (C) and (D). The data represent the mean and standard deviation from at least three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

3.5 Concentration of proline in roots and leaves

Proline acts as an important compatible solute to maintain osmotic homeostasis (Bates, 1973). Accumulation of proline in the salt-stressed leaves and roots of modern cultivar Scarlett and wild barley from Pakistan was measured by Ninhydrin's method. The results were expressed with relative to fresh weight (g).

In Scarlett, the overall amount was higher in leaves than in roots as shown in **Figure 24.** In leaves, the amount increased by 2.2 and 5.9 times when stressed with 180 mM and 275 mM NaCl respectively and the difference were also statistically significant. The amounts

in the roots varied by a positive fold change of 2 and 4.8 times in 180 mM and 275 mM NaCl stressed environments accordingly.

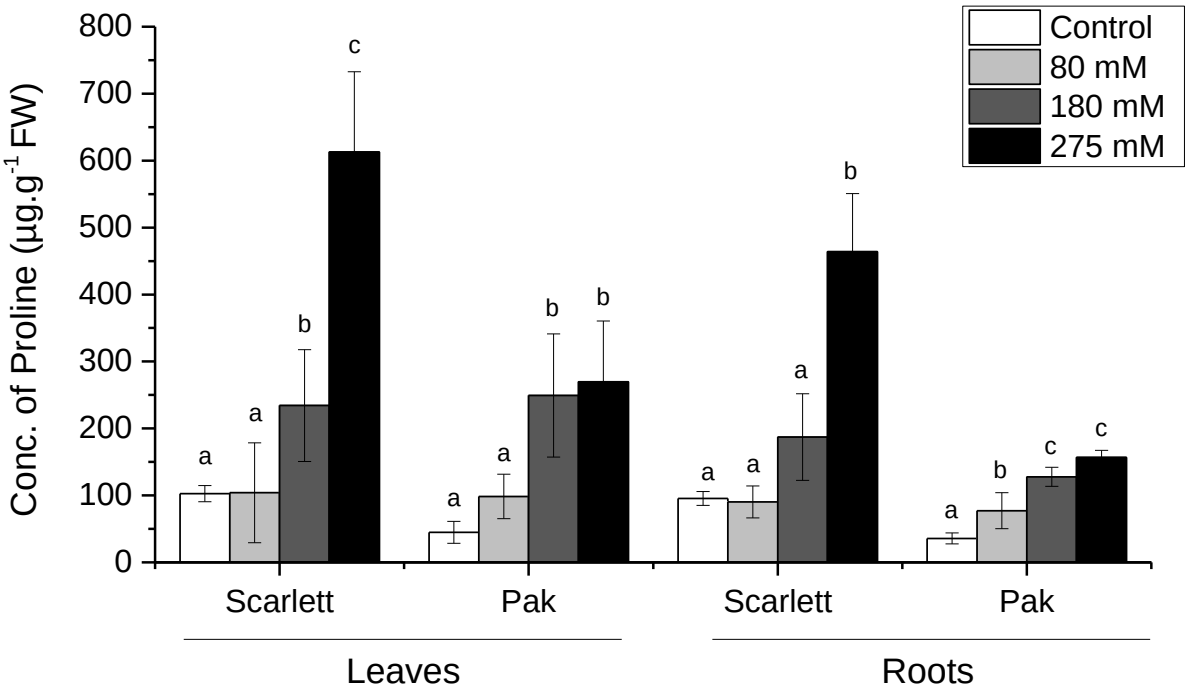

Figure 24 Concentration of proline in leaves and roots of modern cultivar Scarlett and wild barley from Pakistan

The data corresponds to the concentration of proline present in 12d old leaves and seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger apical tip of the root (0-25 %); Zone B (25-50%) and Zone C, matured basal portion of the root (50-100%). The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

The Pakistan wild barley had a significant variation in both leaf and root proline accumulation. There was a linear fold increase of 2.2, 3.6 and 4.3 times in roots treated with 80 mM, 180 mM and 275 mM NaCl respectively (**Figure 24)**. The proline deposition was more pronounced in Scarlett than in wild barley Pakistan especially at the concentration of 275 mM NaCl.

3.6 Osmotic potential in roots

The osmotic potential within the seminal roots of both Scarlett and Pakistan wild barley was measured. There was a decrease in the osmotic potential values with the

increase in the salt concentrations (**Figure 25**). A fold change of 3.4, 5.6 and 6.2 was found in 85 mM, 180 mM and 275 mM salt-stressed roots respectively in both the genotypes and varied significantly.

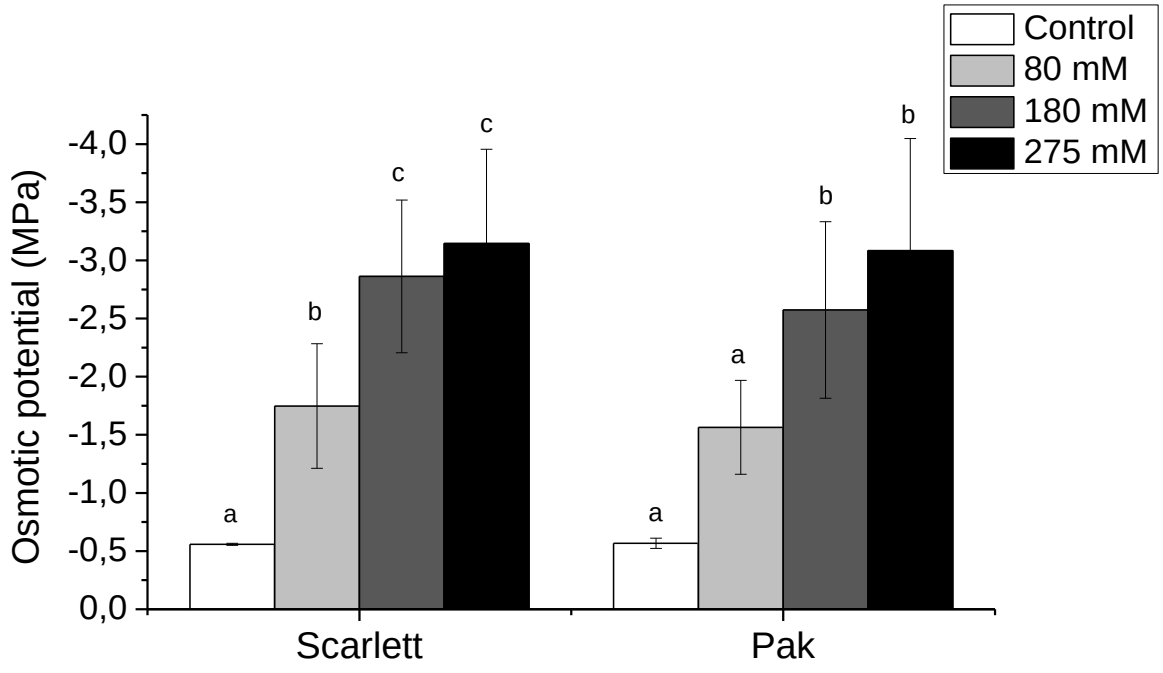

Figure 25 Concentration of osmotic potential in the seminal roots of modern cultivar Scarlett and wild barley from Pakistan

The data corresponds to the concentration of osmotic potential in 12d old seminal roots grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. Zone A represents the younger apical tip of the root (0-25 %); Zone B (25-50%) and Zone C, matured basal portion of the root (50-100%). The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

3.7 Transcriptomics

RNA sequencing analysis done on 0-12.5 % of root length in 12d old Scarlett and wild barley from Pakistan grown in 180 mM salt solution equivalent to water potential -0.8 MPa yielded the following results.

Multi-dimensional scaling (MDS) plot was used to determine transcriptomic relationships between different treatments and genotypes; and also, to emphasize visual groupings of data display. From the plot (**Figure 26**), the clustering of biological replicates based on experimental groups can be identified. The data points were separated based on

the treatment and genotype. The control groups were distinctly placed away from that of stress in addition to four biological replicates closely accumulated.

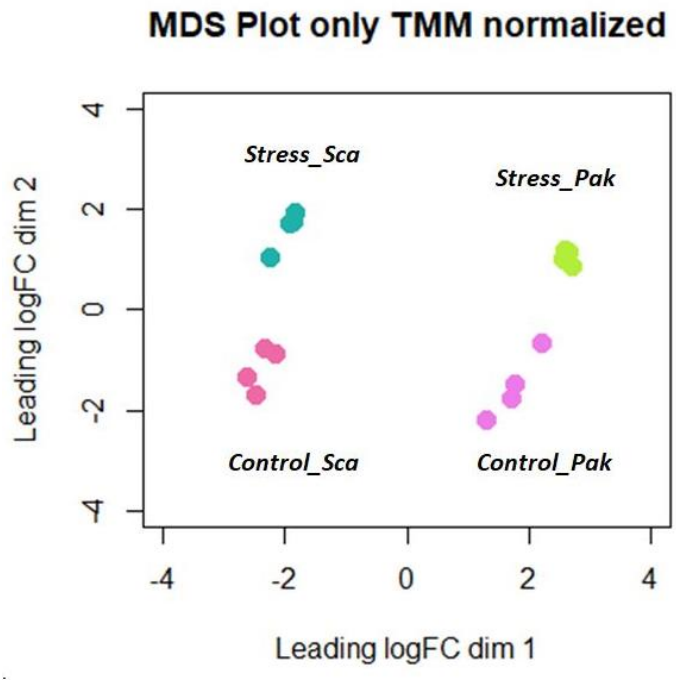

Figure 26 Multi-dimensional scaling plot of seminal roots from RNA sequencing

12d old seminal roots from Scarlett and wild barley from Pakistan were grown in control and 180 mM NaCl (equivalence of water potential – 0.8 MPa). The four biological replicates for the RNA sequencing samples were obtained from 0-12.5 % of the root length.

Volcano plot shows the -log10 q-value of genes on the y-axis as a measure of statistical significance and log fold change on the x-axis (**Figure 27**). The number of differentially expressed genes between the control and stressed treatments was shown in **Figure 28**. There was an overall of 3737 unique DEGs up regulated and 3833 DEGs down regulated when compared between stress and control conditions in cultivar Scarlett and wild barley from Pakistan. In Scarlett, 2374 genes were up regulated and 891 genes were down regulated. In case of Pakistan wild barley, 853 genes were up regulated and 2648 genes were down regulated. Around 851 genes were differentially expressed in both genotypes.

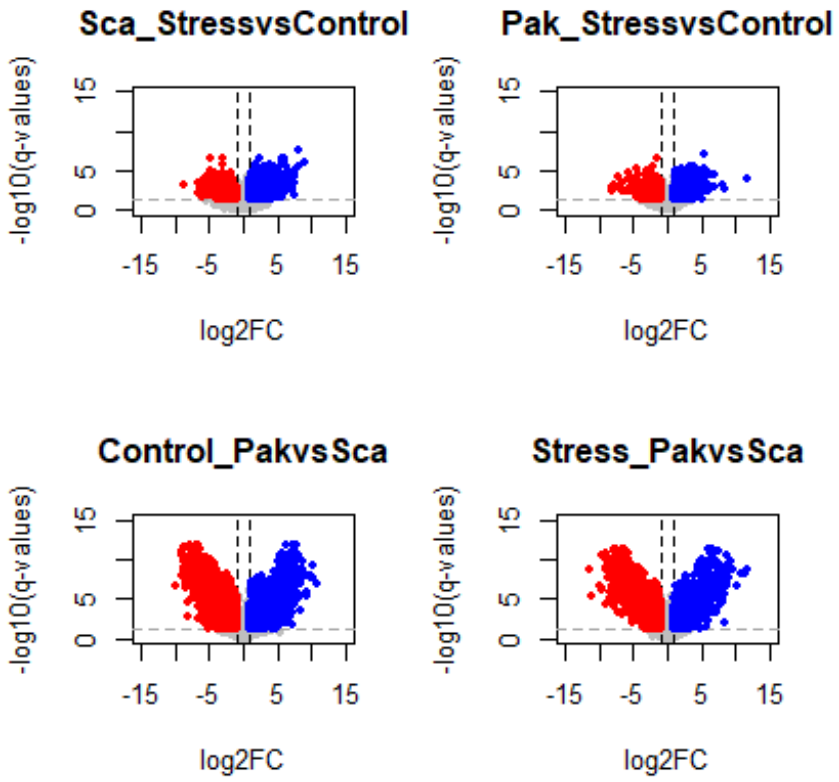

Figure 27 Depiction of differentially expressed genes (DEGs) by volcano plots

Up regulated DEGs were represented in blue dots and down regulated DEGs in red dots. DEGs that did not exceed the threshold of $|log_2FC| > 1$ and FDR ≤1% are depicted as grey dots.

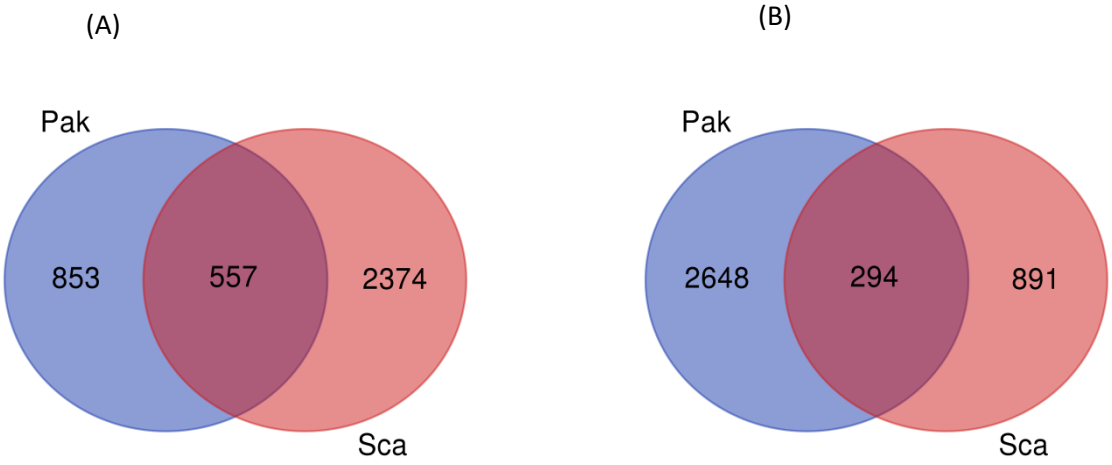

Figure 28 Venn diagram showing the overlap of DEGs between Scarlett and wild accession from Pakistan

Cross comparisons between the control and salt stress treatments were depicted in the Venn diagram. (A) Number of DEGs up regulated and (B) number of DEGs down regulated in both the genotypes.

Differential expressions of genes involved in suberin biosynthesis were analysed. Putative key genes that play a critical role in suberin biosynthesis and transport were positively regulated. The functional role of these genes has been elaborated in section **1.4**. The log_2FC expression for each gene was shown in **Figure 29**.

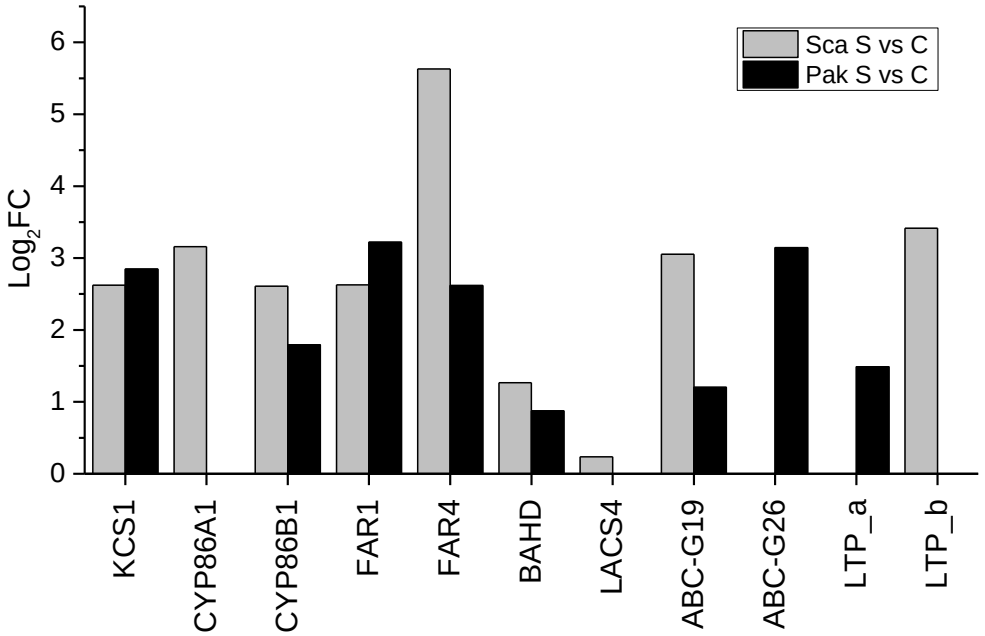

Figure 29 Expression of suberin biosynthesis genes in the seminal roots of Scarlett and wild barley from Pakistan

Each data represents the log_2FC expression at a significance level of 0.05 in pairwise t-tests between the roots grown in control and 180 mM NaCl. Four biological replicates were used for each treatment.

Major suberin biosynthesis genes like KCS1-ß Ketoacyl-CoA synthase (HORVU4Hr1G063420), CYP86B1-Cytochrome P450-dependent ω-hydroxylases (HORVU1Hr1G042810), FAR1-Fatty acyl CoA reductase (HORVU7Hr1G020270), FAR4 (HORVU4Hr1G001450), BAHD (HORVU2Hr1G035810) were up regulated in both the genotypes; whereas CYP86A1 (HORVU3Hr1G085020), LACS4-Long chain Acyl CoA Synthetase (HORVU3Hr1G059140) were differentially expressed only in Scarlett. Genes related to family of ATP Binding Cassette Transporters, ABC-G19 (HORVU1Hr1G009920), ABC-G26 (HORVU7Hr1G090350) and Lipid transport proteins; LTPs (HORVU2Hr1G102100 and HORVU2Hr1G102110) were also positively regulated.

Additionally, genes involved in aquaporin regulation were also differentially expressed. The main function of these families of genes was to facilitate the movement of

water and solutes across the cell membrane (Quigley *et al*., 2002). From **Figure 30**, it was evident that the expression was more prevalent in Pakistan wild barley roots than in Scarlett. Amongst the family of Plasma intrinsic proteins (PIPs) and Tonoplast intrinsic proteins (TIPs), only PIP2; 1_d (HORVU2Hr1G089970) and TIP 3; 1_b (HORVU3Hr1G094900) were differentially regulated in Scarlett. Other families of aquaporins (NIPs and SIPs) were not differentially expressed in both Scarlett and Pakistan wild barley.

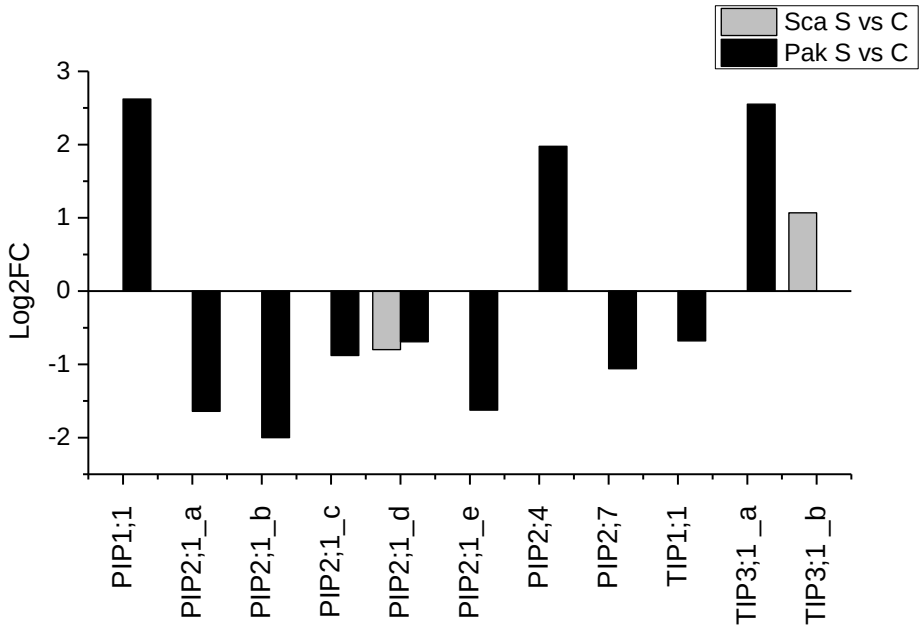

Figure 30 Expression of aquaporin genes in the seminal roots of Scarlett and wild barley from Pakistan

Each data represents the log_2FC expression at a significance level of 0.05 in pairwise t-tests between the roots grown in control and 180 mM NaCl. Four biological replicates were used for each treatment

Some of the genes found to be involved in the salt stress signalling pathway were also distinctly regulated (**Figure 31**). NHX1 - vacuolar Na^+/H^+ antiporter (HORVU2Hr1G021020) ; HKT - high-affinity K^+ transporter (HKT1-2 - HORVU0Hr1G022090 ; HKT3- HORVU7Hr1G096920) , putative orthologs from *Oryza sativa* involved in regulation of K^+/Na^+ homeostasis (Horie *et al*., 2001) were down regulated. CBL4 - Calcineurin B-like protein 4 (HORVU1Hr1G080820) involved in the regulatory pathway for the control of intracellular Na^+ and K^+ homeostasis and salt tolerance (Martínez-Atienza *et al*., 2007) and CIPK24 - CBL-interacting protein kinase 24 (HORVU7Hr1G090260) that phosphorylates Ca^{2+} dependent CBL proteins and thereby activating regulatory stress responsive pathways (Cheng *et al*., 2004) were also differentially expressed.

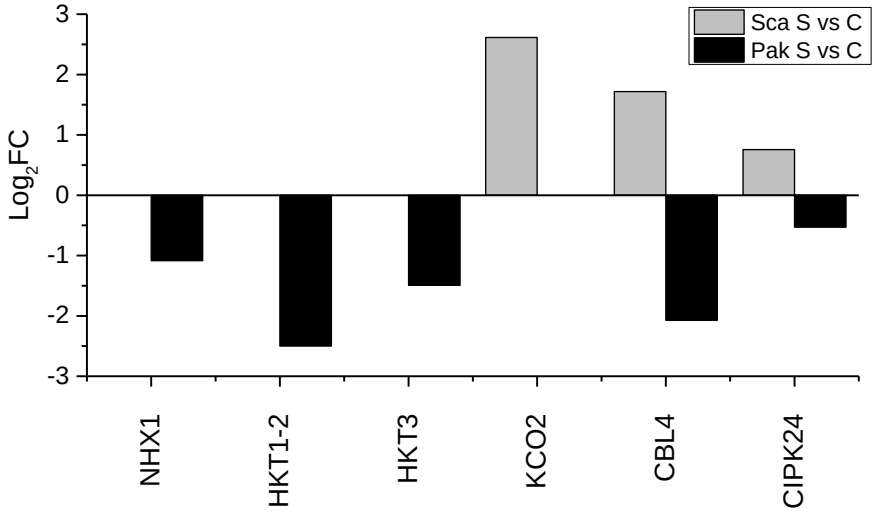

Figure 31 Expression of salt stress related genes in the seminal roots of Scarlett and wild barley from Pakistan

Each data represents the log₂FC expression at a significance level of 0.05 in pairwise t-tests between the roots grown in control and 180 mM NaCl. Four biological replicates were used for each treatment

Osmoprotectants such as proline play an integral function in maintaining ion homeostasis during stress environments. HORVU1Hr1G072780 gene that encodes P5CS (Pyrroline-5-carboxylate synthase 1); plays a key role in proline biosynthesis (Dong *et al*., 2010) and was found to be positively regulated in both Scarlett and Pakistan wild barley roots (**Figure 32**).

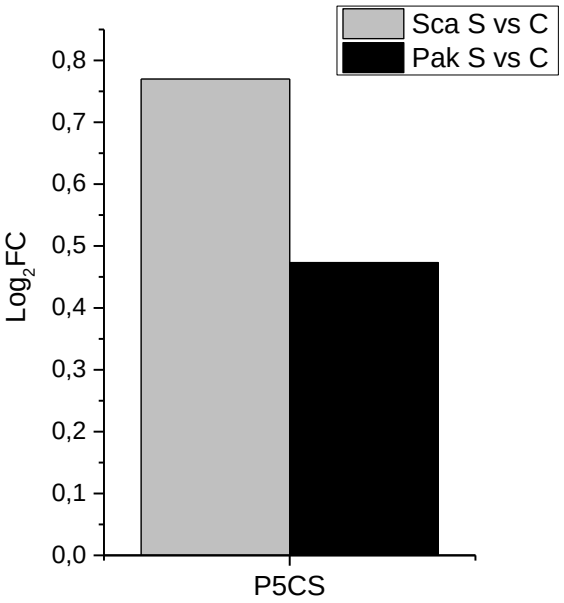

Figure 32 Expression of proline biosynthesis genes in the seminal roots of Scarlett and wild barley from Pakistan

Each data represents the log₂FC expression at a significance level of 0.05 in pairwise t-tests between the roots grown in control and 180 mM NaCl. Four biological replicates were used for each treatment

3.7.1 Gene ontology studies

To characterize the functional annotation of proteins, gene ontology terms were designated to DEGs. GO terms in general, had been broadly categorized into – molecular function, biological process and cellular component. Singular enrichment analysis (SEA) compared the annotations in the differentially expressed gene (DEG) list against a population of background genes via AgriGO v2.0, a web-based tool and database for gene ontology analyses. The outcome of SEA analyses was subsequently used for cross-comparisons using the SEACOMPARE tool.

GO terms only with FDR < 5% was retained to filter out redundant units. In Scarlett, a total of 155 (47 up regulated and 108 down regulated) GO terms were significantly enriched whereas in Pakistan wild barley only 29 (22 up regulated and 7 down regulated) terms were expressed significantly (**Table S1 and Table S2**).

Some of the following significant GO terms were strongly enriched in 180 mM salt-stressed roots of both Scarlett and wild barley from Pakistan. GO terms relating to biological process, 'protein modification process' (GO: 0036211), 'protein phosphorylation' (GO: 0006468) were found. GO terms categorised as molecular function like 'catalytic activity' (GO:0003824) including 'kinase' (GO:0016301), 'transferase' (GO:0016740) and 'hydrolase' (GO:0004553) were also up regulated. 'Calcium ion binding' (GO: 0005509), 'phosphorylation' (GO: 0016310) that could potentially play an indispensable role in salt stress-responsive pathways were also found. 'Regulation of gene expression' (GO: 0010468) and its relative function 'Transcription factor activity' (GO: 0003700) were also enhanced. 'Regulation of biosynthetic process' (GO: 2001141) was exclusively found in the cultivar, Scarlett. GO terms associated with binding affinity to nucleotide (GO: 0000166), ATP (GO: 0005524), protein (GO: 0005515), carbohydrate (GO: 0097367) and ion (GO: 0043167) were significantly enriched only in Scarlett.

The GO terms that were negatively regulated collectively coin to the cellular component part of gene ontology. 'Intracellular membrane-bounded organelle' (GO: 0043231) and 'membrane-bounded organelle' (GO: 0043227) were found in both the genotypes, however strongly regulated in Scarlett than in Pakistan wild barley. Additionally, 'metabolic process' (GO: 0019222) and 'biosynthetic process' GO: 0009058 were down

regulated in Scarlett. Cellular developmental GO terms– 'cellular component biogenesis' (GO: 0044085), 'cellular component assembly' (GO: 0022607), 'cellular metabolic process' (GO: 0044237) and 'intracellular transport' (GO: 0046907) were down regulated in Scarlett. GO term defined for 'protein binding' (GO: 0005515) was downregulated in Pakistan wild barley. Comprehensively, the significant GO terms were strongly enhanced in cultivar Scarlett than in wild accession from Pakistan.

3.8 Stomatal conductance of leaves

The rate of stomatal transpiration in the leaves was measured in terms of conductance. The measurement was taken briefly after the implementation of stress. The concentrations of salt 80 mM, 180 mM and 275 mM caused significant decrement in Scarlett (**Figure 33** A) and in wild barley Pakistan (**Figure 33** B). After the initial drop in the values in 30 minutes, the measurement was subsequently carried out for two days, within which a steady state was maintained by the stressed leaves to prevent further transpiration. In Scarlett there was a decrease by 1.5, 2.6 and 2.7 times in 80 mM, 180 mM and 275 mM stressed leaves respectively after 30 minutes of stress period. In case of Pakistan wild barley, the values reduced by 1.7, 2.4 and 3.7 times in 80 mM, 180 mM and 275 mM stressed leaves respectively after 30 minutes. At the end of the measurement, a maximum fold decrease of 2.6 and 4.3 was observed in Scarlett and Pakistan wild type respectively in 275 mM NaCl stressed leaves.

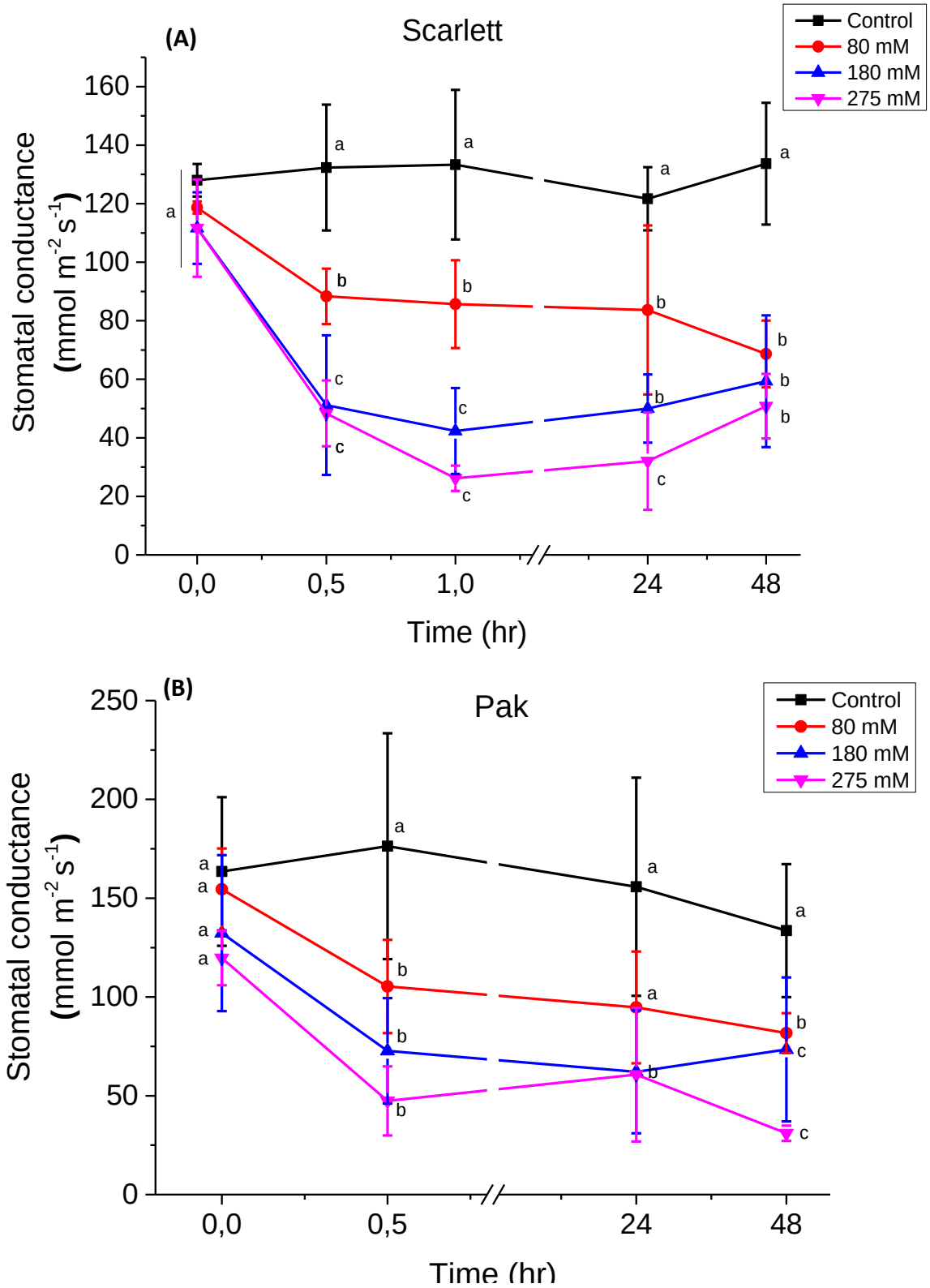

Figure 33 Measurement of stomatal conductance in the leaves of (A) cultivar Scarlett and (B) wild barley from Pakistan

The leaves were grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. The measurements were made on the adaxial side of the leaves. The data represent the mean and standard

deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

3.9 Photosynthetic yield measurement

The impact of salinity on photosynthesis was measured in terms of the light curve. The results were obtained in the form of photosynthetic yield from photosystem - II (PS-II) and Electron transport rate (ETR). Pulse radiation of range 25-820 (µmol m^{-2} s^{-1}), incident on the leaves yielded the following outcomes. In both genotypes, on the first day of stress application, there was no difference between the control and stressed plants. However, as the prolongation of the stress occurs, there was a drop in the photosynthetic yield on day 3 (**Figure 34**). This change was also reflected in ETR values (**Figure 35**). The difference was sharper in wild barley of Pakistan accession with a significant difference especially at higher salt concentration of 180 mM and 275 mM.

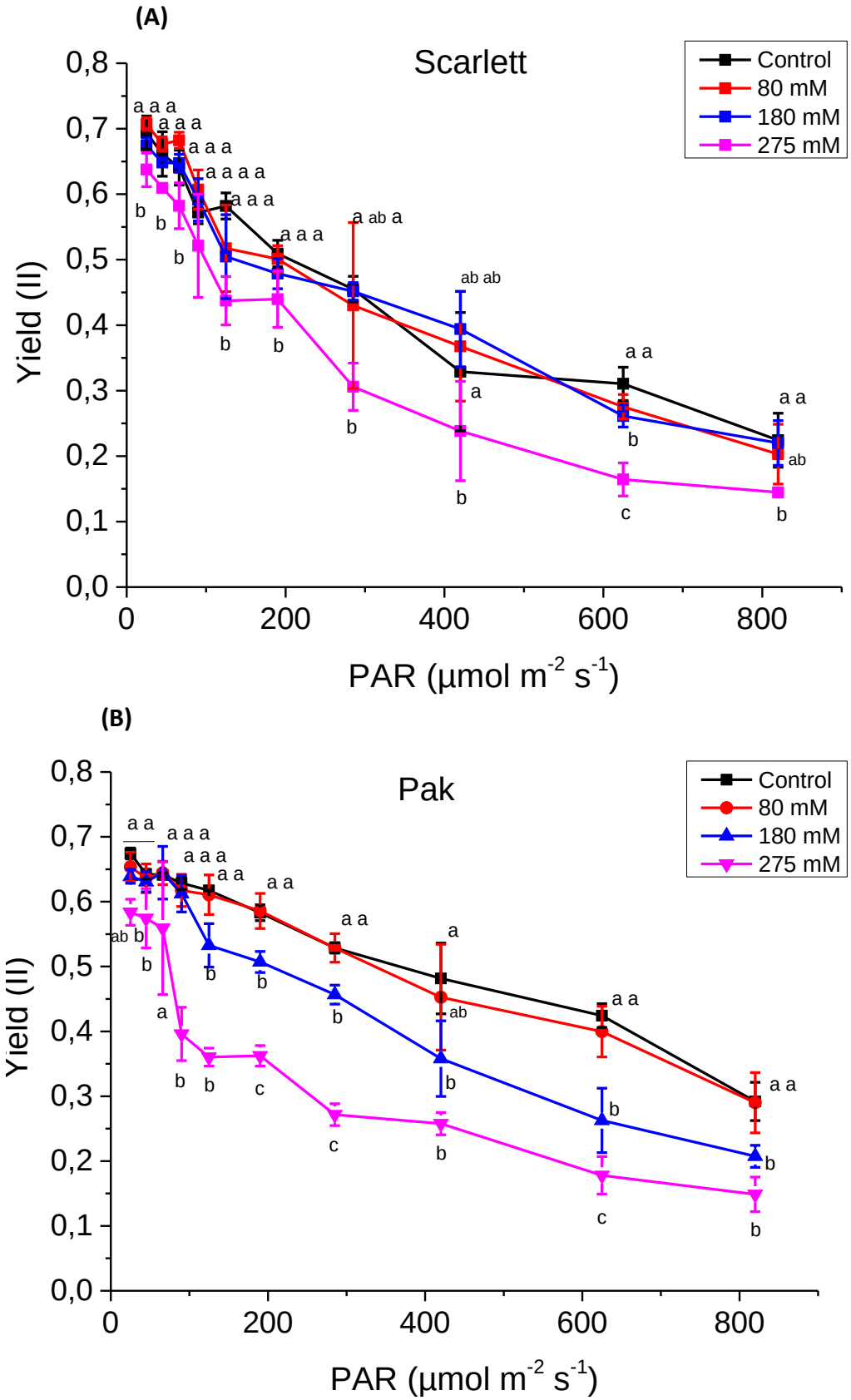

Figure 34 Light curves for salt-stressed barley leaves (A) Scarlett and (B) Pakistan wild barley

The leaves were grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. The

measurements were made on the adaxial side of the leaves. The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

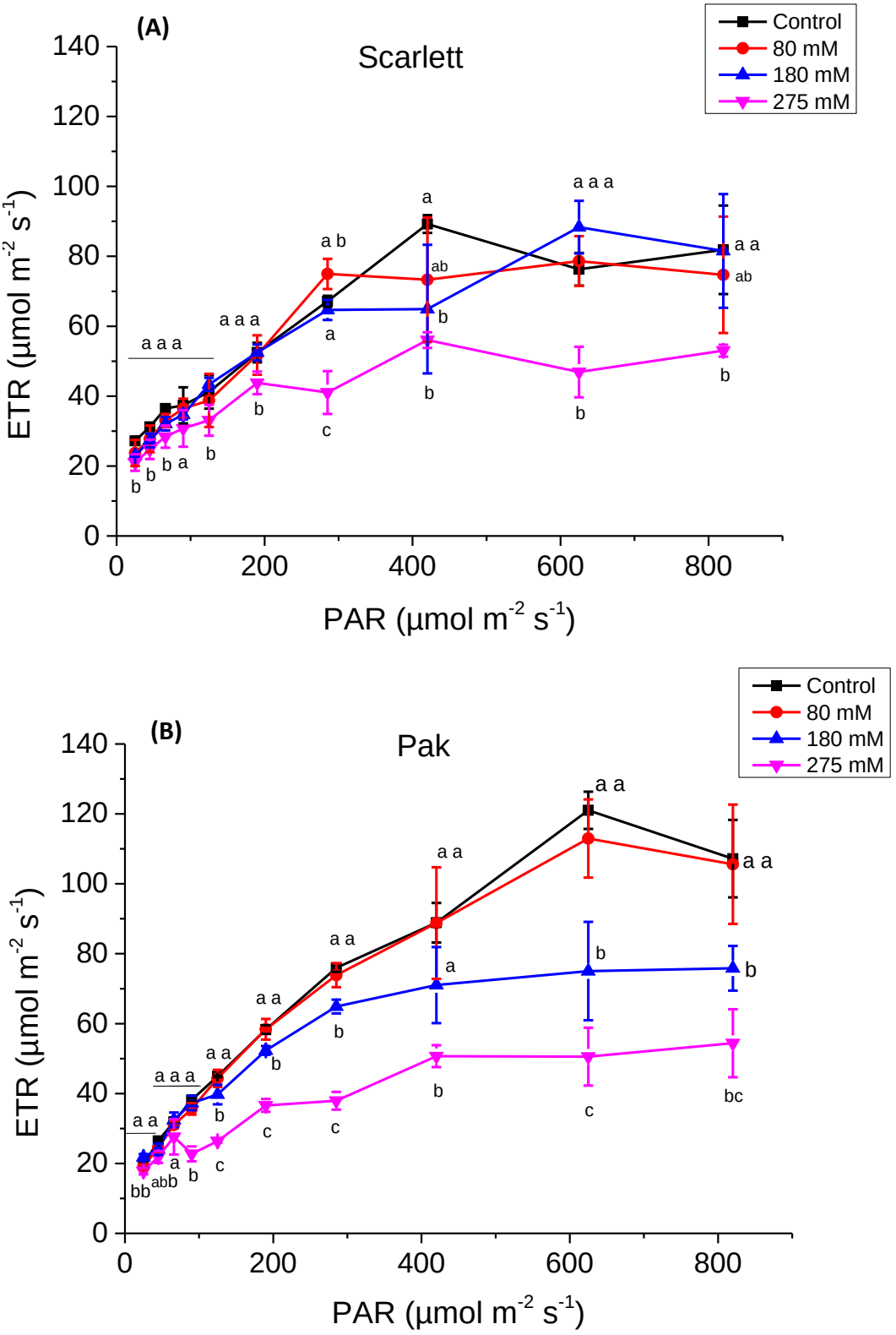

Figure 35 Electron transport rates (ETR) for salt-stressed barley leaves (A) Scarlett and (B) Pakistan wild barley

The leaves were grown in control and different salt stress concentrations of 80 mM, 180 mM and 275mM NaCl which corresponds to a water potential equivalent of -0.4 MPa, -0.8 MPa and -1.2 MPa respectively. The measurements were made on the adaxial side of the leaves. The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

3.10 Wax and cutin analytics

Both wax and cutin analysis were done in 12d old leaves of cultivar Scarlett and wild barley from Pakistan. The plants were grown in control and a stress solution treated with 180 mM NaCl which corresponds to the water potential of – 0.8 MPa. Leaf 1 (first developed older leaf) and Leaf 2 (younger one) were used for GC analysis. The amounts were expressed with relative to leaf surface area (cm^{-2})

3.10.1 Chemical analytics of wax

The total wax amounts of Scarlett and Pakistan wild barley were shown in **Figure 36**. In Scarlett, the concentration of wax did not vary between the control and stress treatments in both leaf 1 and leaf 2 (**Figure 36** A). On the contrary, wild accession barley from Pakistan had a significant increase in the wax amounts in leaf 2, with a fold change of 3.3 (**Figure 36** B).

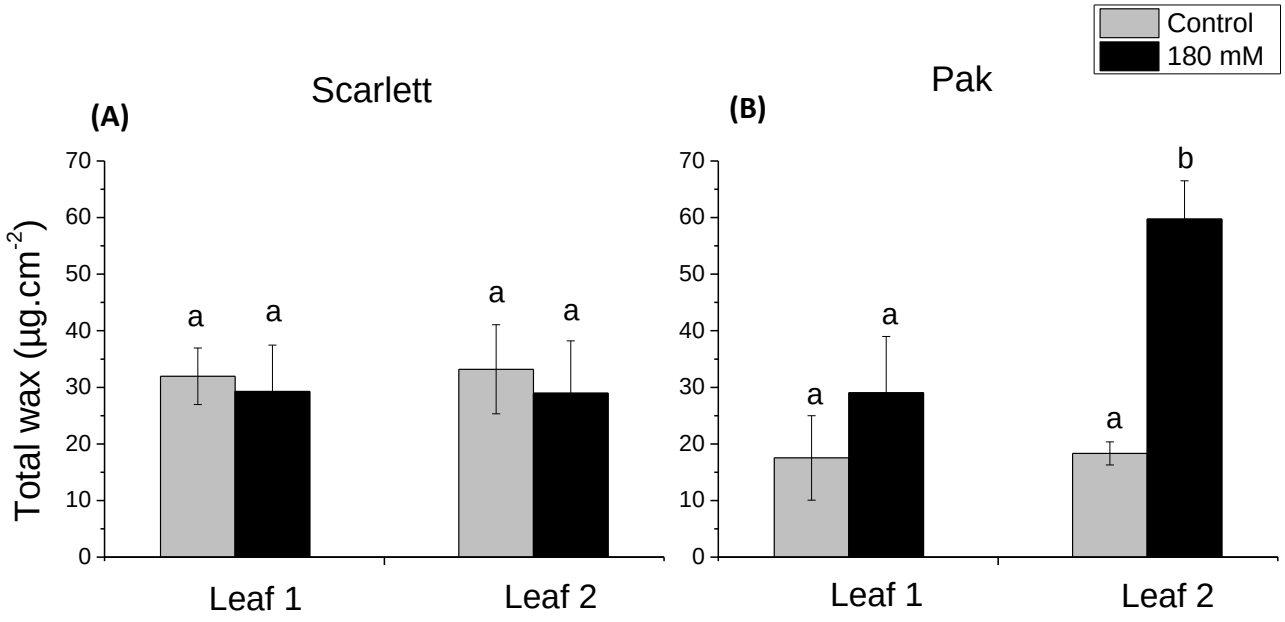

Figure 36 Total wax concentration per unit area (µg.cm^{-2}) in the leaves of (A) barley cultivar Scarlett and (B) wild barley from Pakistan

The data corresponds to the concentration of total wax present in 12d old leaf 1 and leaf 2, grown in control and salt stress concentration of 180 mM NaCl which corresponds to a water potential equivalent of -0.8 MPa. The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

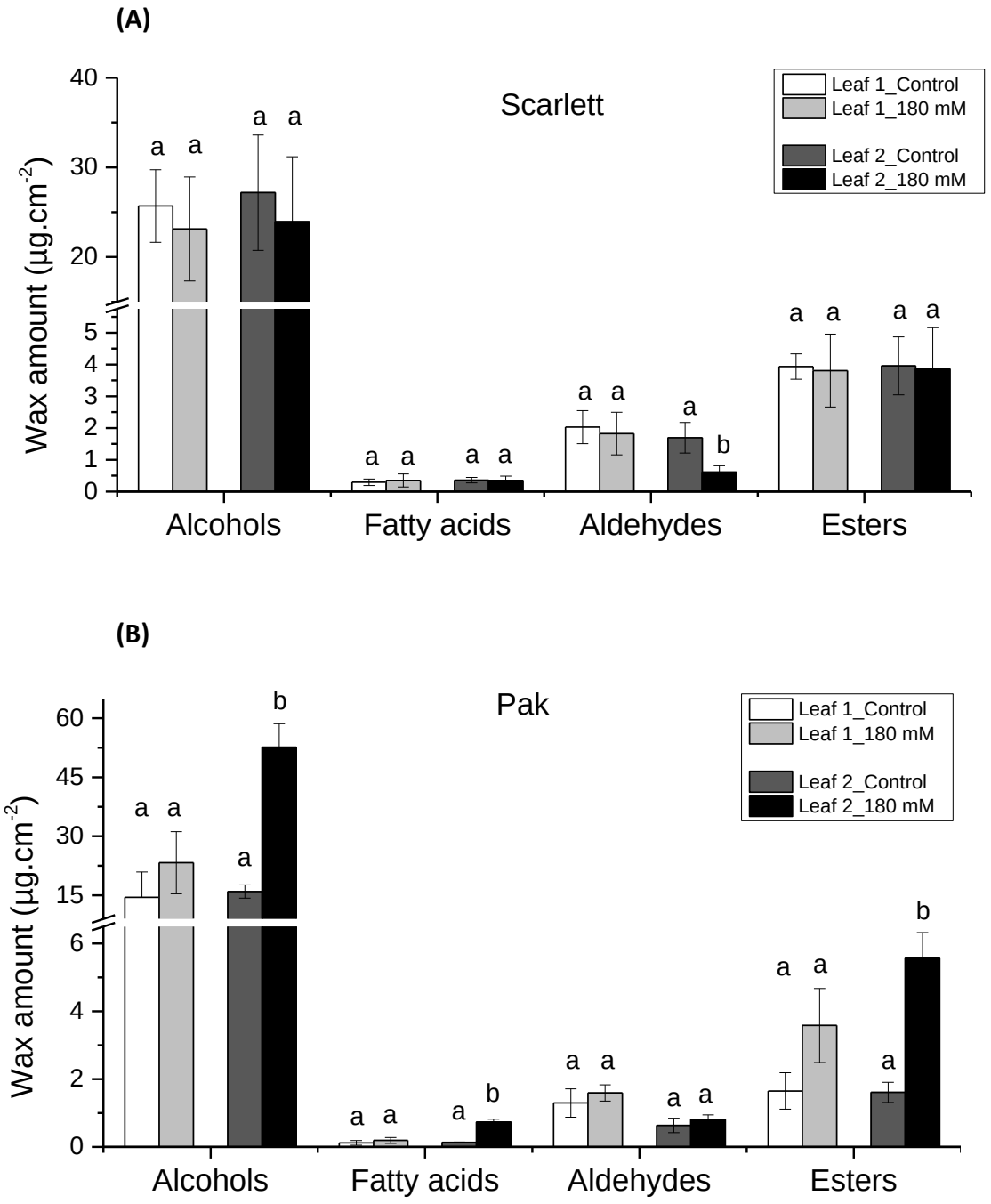

Figure 37 Wax substance classes concentration per unit area (µg.cm^{-2}) in the leaves of (A) barley cultivar Scarlett and (B) wild barley from Pakistan

The data corresponds to the concentration of wax substance classes present in 12d old leaf 1 and leaf 2, grown in control and salt stress concentration of 180 mM NaCl which corresponds to a water potential equivalent of -0.8 MPa. The data represent the mean and standard deviation from three biological replicates; Different

alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

The wax classes identified were similar for the leaves in both genotypes. The constituents of wax classes include- alcohols, fatty acids, aldehydes and esters (**Figure 37**). Alcohols were the major contributor followed by esters, aldehydes and fatty acids in that order in both the genotypes. The wax class's amount in modern cultivar Scarlett (**Figure 37** A) did not vary significantly in both leaf 1 and leaf 2 except for the aldehydes content in stressed leaf 2 with a fold decrease of 0.36. However in wild barley from Pakistan, the deposition of wax classes varied significantly in alcohols, fatty acids and esters of stressed leaf 2 with a positive fold change of 3.3; 5.6, and 3.5 respectively (**Figure 37** B).

The monomeric compositions of the two genotypes were shown in **Figure 38** and **Figure 39**. The chain lengths varied between C_{20} to C_{28} in alcohols; C_{22} to C_{26} in fatty acids; C_{26} to C_{28} in aldehydes and C_{38} to C_{50} in esters and this deposition pattern was identical in Scarlett as well as in Pakistan wild barley. In Scarlett (**Figure 38)**, C_{26} alcohol was found abundantly with a maximum concentration of 22.52 ± 6.7 $\mu g.cm^{-2}$. Also, C_{22} alcohols varied significantly by a factor of 3.8 and 1.4 in both leaf 1 and leaf 2 respectively. C_{26} fatty acids contributed as the highest among its class with a range of 0.18 ± 0.06 $\mu g.cm^{-2}$ to 0.25 ± 0.14 $\mu g.cm^{-2}$ in stressed samples. The aldehyde class differed strongly in leaf 2. Esters, the second most contributors in the wax class group, did not vary in amounts between the treatments. C_{46} ester was predominantly found at a value of 1.47 ± 0.35 $\mu g.cm^{-2}$. Among the monomers in Pakistan wild barley (**Figure 39)**, C_{26} alcohol in stressed leaf 2 increased with a maximum fold increase of 3.2. The amounts in stressed leaf 2 varied distinctively by a factor of 3.0; 5.8 and 6.3 times in C_{22}, C_{24}, and C_{26} fatty acids accordingly. The monomers of aldehydes and esters also significantly increased in leaf 2 stressed with 180 mM NaCl.

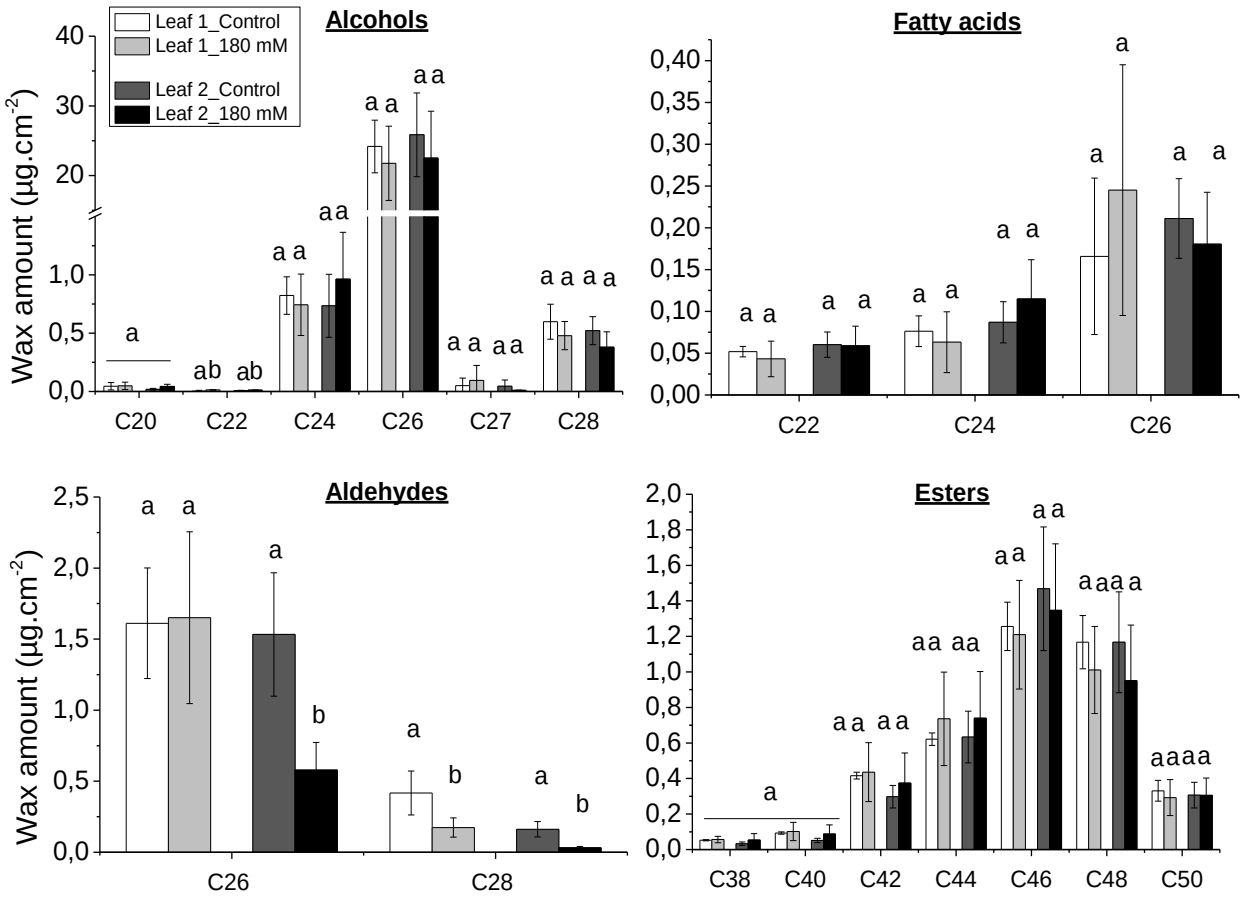

Figure 38 Wax monomers concentration per unit area (µg.cm^{-2}) in the leaves of barley cultivar Scarlett

The data corresponds to the concentration of wax monomers present in 12d old leaf 1 and leaf 2, grown in control and salt stress concentration of 180 mM NaCl which corresponds to a water potential equivalent of -0.8 MPa. The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

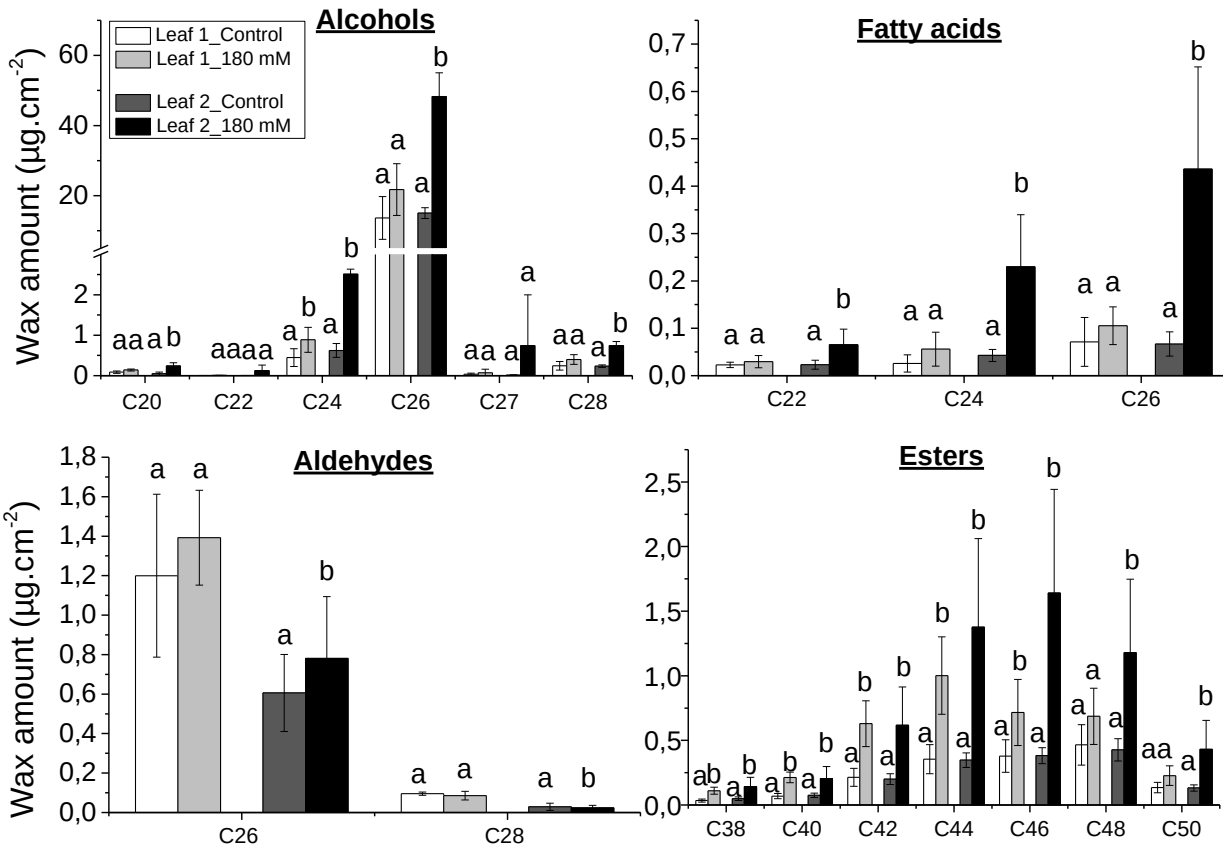

Figure 39 Wax monomers concentration per unit area (µg.cm^{-2}) in the leaves of wild barley from Pakistan

The data corresponds to the concentration of wax monomers present in 12d old leaf 1 and leaf 2, grown in control and salt stress concentration of 180 mM NaCl which corresponds to a water potential equivalent of -0.8 MPa. The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

The concentration of total wax per leaf (µg) yielded a change in the graphs as seen in **Figure 40**. In both the genotypes, the amount of total wax in leaf 2 decreased significantly in stressed leaf 2. This ambiguity could be due to the detrimental developmental effect of leaf 2 in the salt solution.

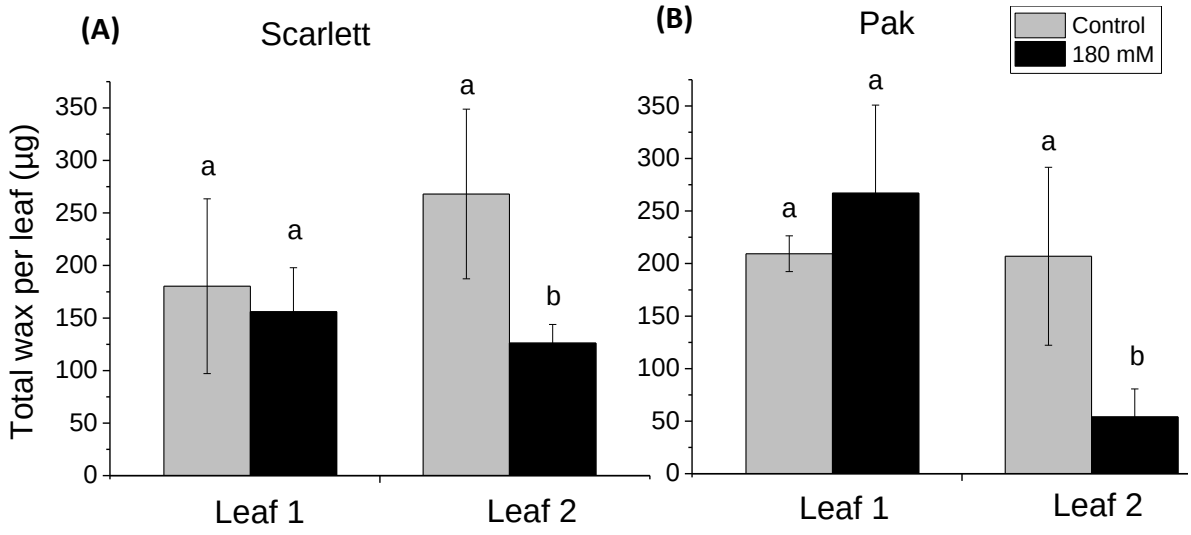

Figure 40 Total wax concentration per leaf (µg) in (A) barley cultivar Scarlett and (B) wild barley from Pakistan

The data corresponds to the concentration of total wax present in 12d old leaf 1 and leaf 2, grown in control and salt stress concentration of 180 mM NaCl which corresponds to a water potential equivalent of -0.8 MPa. The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 level in one way ANOVA (Fisher's least significant difference, LSD).

3.10.2 Chemical analytics of cutin

The quantitative and qualitative cutin amounts in 12 d old barley leaves were assessed in both the genotypes. The total amount of cutin extracted did not differ between the stress and control conditions in leaf 1 but in leaf 2 there was a fold increase of 2.4 in Scarlett and also in Pakistan wild barley with a maximum concentration of 17.44 ± 3.62 µg.cm^{-2} (**Figure 41**).

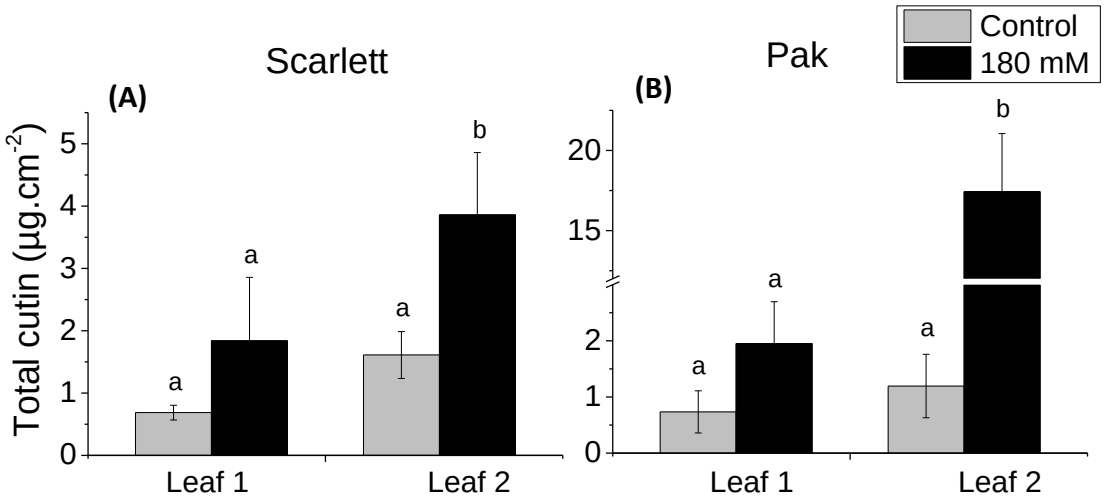

Figure 41 Total cutin concentration per unit area (µg.cm^{-2}) in the leaves of (A) barley cultivar Scarlett and (B) wild barley from Pakistan

The data corresponds to the concentration of total cutin amounts present in 12d old leaf 1 and leaf 2, grown in control and salt stress concentration of 180 mM NaCl which corresponds to a water potential equivalent of -0.8 MPa. The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 level in one way ANOVA (Fisher's least significant difference, LSD).

Cutin monomer classes were identified and included, groups of fatty acids, ω-hydroxy acids and 9, 10-epoxy-18-hydroxy-stearic acids. Amongst these classes in Scarlett (**Figure 42** A), 9, 10-epoxy C_{18} OH were found in the highest amounts followed by ω- OH acids and varied significantly in concentration with a fold increase of 2.9 and 3.3 times only in the stressed leaf 2. In Pakistan wild barley also, a similar pattern was observed (**Figure 42** B). However in stressed leaf 2, the amounts of fatty acids, ω- OH acids and 9, 10-epoxy-18-hydroxy-stearic acids differed significantly with a concentration of 1.48 ± 0.07 µg.cm^{-2}, 4.07 ± 0.88 µg.cm^{-2} and 7.83 ± 2.12 µg.cm^{-2}respectively.

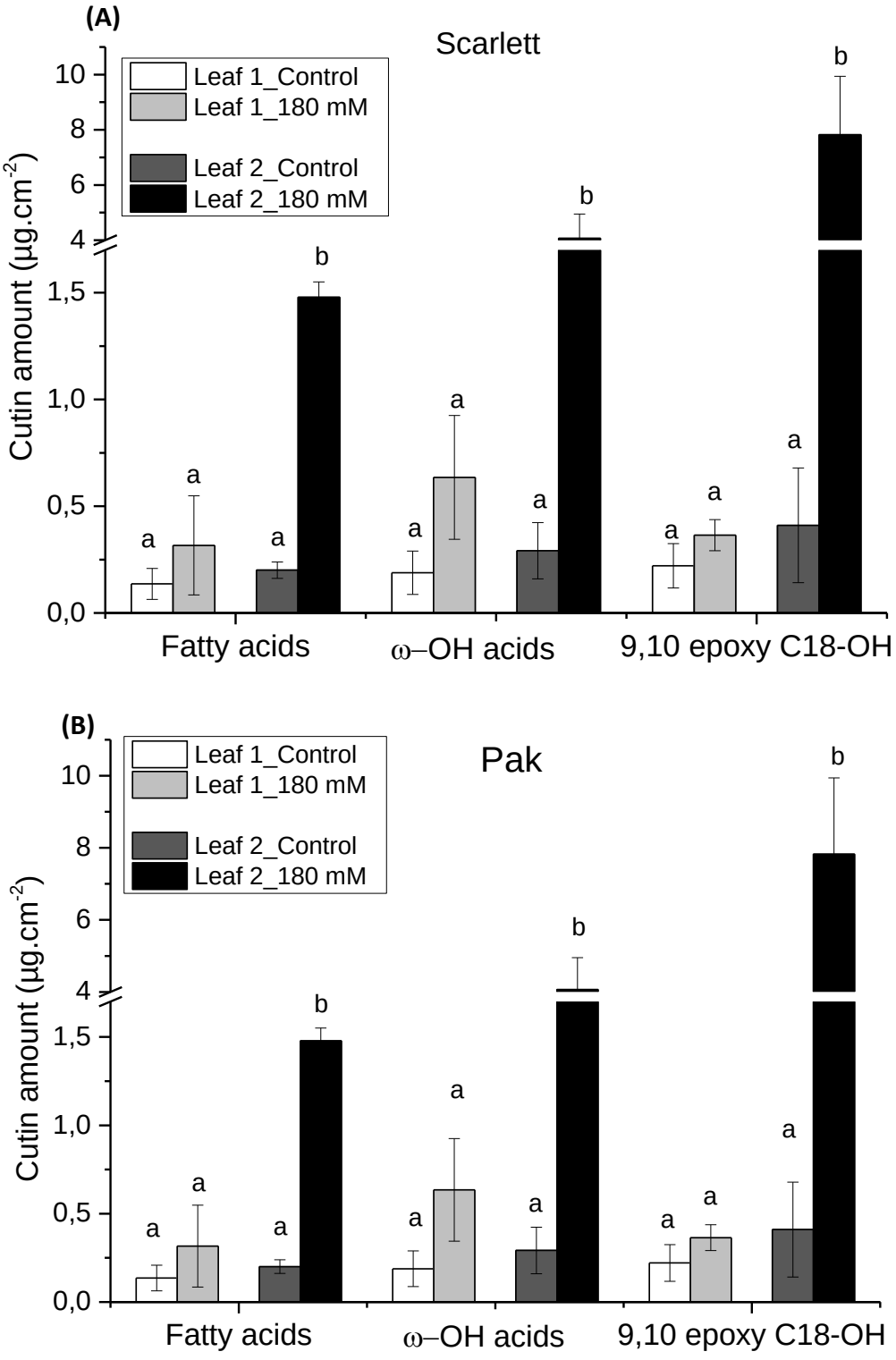

Figure 42 Cutin substance classes concentration per unit area (µg.cm^{-2}) in the leaves of (A) barley cultivar Scarlett and (B) wild barley from Pakistan

The data corresponds to the concentration of cutin substance classes present in 12d old leaf 1 and leaf 2, grown in control and salt stress concentration of 180 mM NaCl which corresponds to a water potential equivalent of -0.8 MPa. The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

The chain lengths in monomers varied between C_{18} and C_{24} in fatty acids; C_{16} to C_{24} in ω- OH acids; and only C_{18} in 9, 10-epoxy C_{18} OH in both the genotypes. The concentration of $C_{18:1}$ ω- OH acid and 9, 10-epoxy C_{18} OH was found in abundance with an amount of 1.44 ± 0.67 µg.cm^{-2} and 2.51 ± 0.05 µg.cm^{-2} in Scarlett (**Figure 43**) and 3.32 ± 0.8 µg.cm^{-2} and 7.82 ± 2.11 µg.cm^{-2} in Pakistan wild barley (**Figure 44**) respectively.

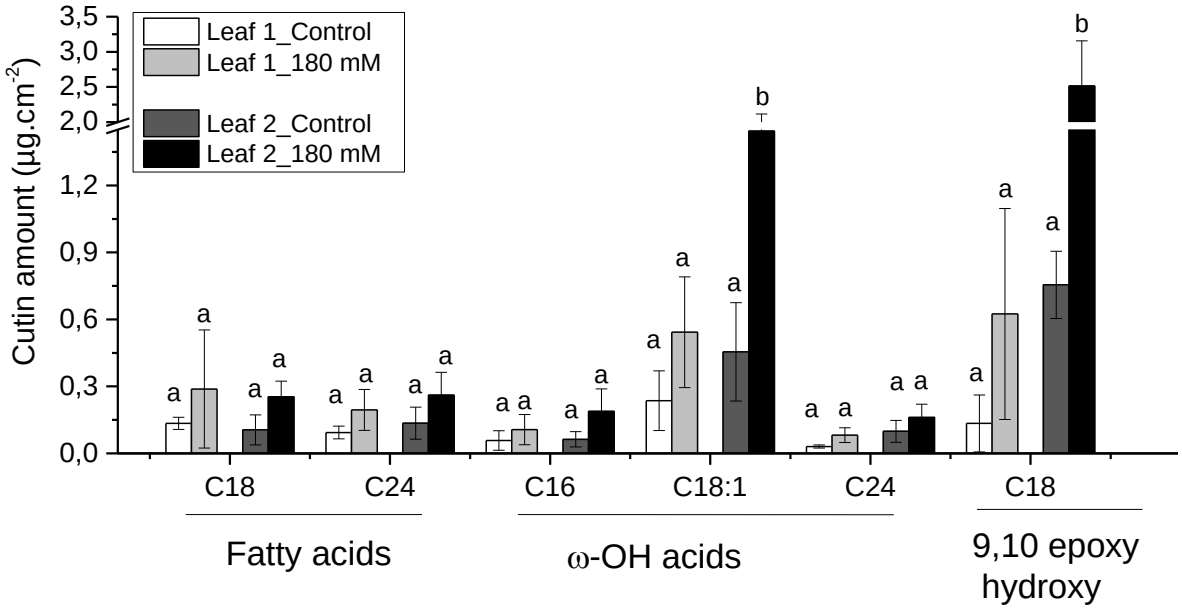

Figure 43 Cutin monomers concentration per unit area (µg.cm^{-2}) in the leaves of barley cultivar Scarlett

The data corresponds to the concentration of cutin monomers present in 12d old leaf 1 and leaf 2, grown in control and salt stress concentration of 180 mM NaCl which corresponds to a water potential equivalent of -0.8 MPa. The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

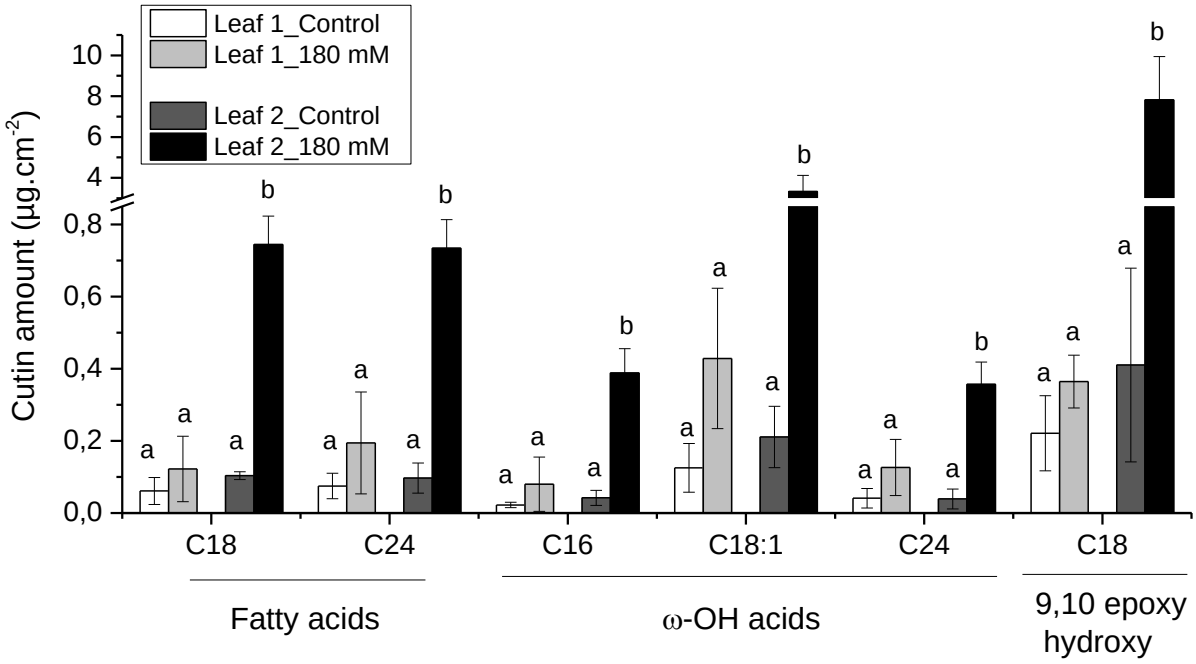

Figure 44 Cutin monomers concentration per unit area (µg.cm^{-2}) in the leaves of wild barley from Pakistan

The data corresponds to the concentration of cutin monomers present in 12d old leaf 1 and leaf 2, grown in control and salt stress concentration of 180 mM NaCl which corresponds to a water potential equivalent of -0.8 MPa. The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

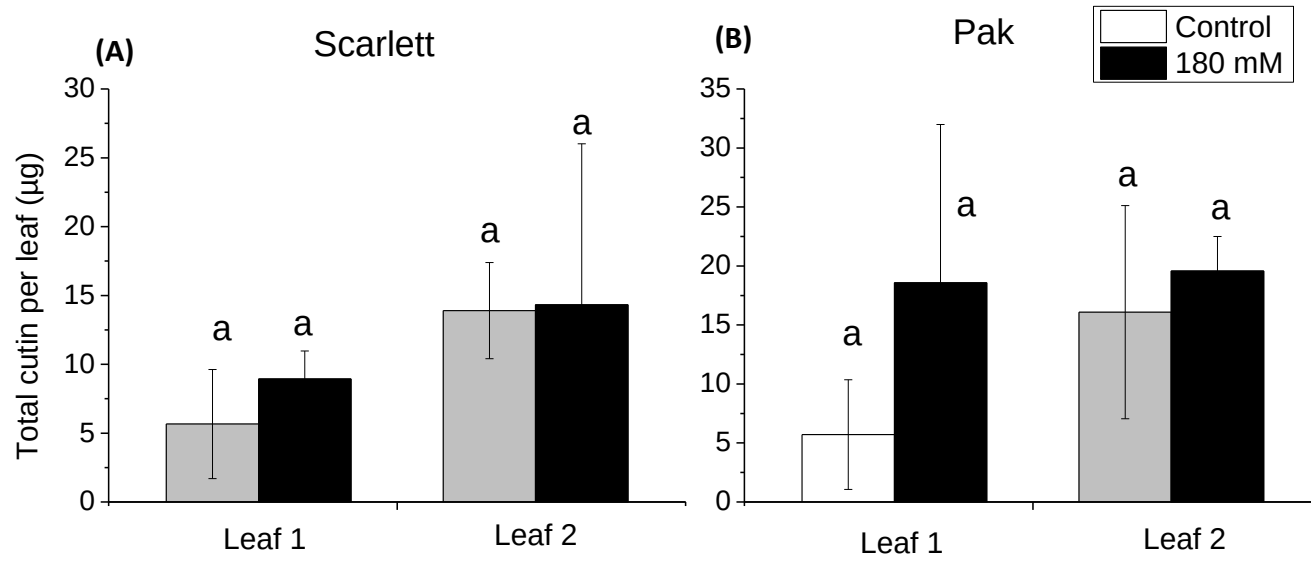

Figure 45 Total cutin concentration per leaf (µg) in (A) barley cultivar Scarlett and (B) wild barley from Pakistan

The data corresponds to the concentration of cutin monomers present in 12d old leaf 1 and leaf 2, grown in control and salt stress concentration of 180 mM NaCl which corresponds to a water potential equivalent of -0.8 MPa. The data represent the mean and standard deviation from three biological replicates; Different alphabet denotes significant differences between the means at 0.05 levels in one way ANOVA (Fisher's least significant difference, LSD).

Similar to wax, the concentration of total cutin per leaf (µg) yielded a change in the graphs as seen in **Figure 45**. In both the genotypes, the amount of total cutin did not differ significantly in stressed leaf 2 in contrary to that expressed in $\mu g.cm^{-2}$. This ambiguity could be due to the detrimental developmental effect of leaf 2 in the salt solution.

4. DISCUSSION

Differential responses of 12d old barley plants at varying degree of salt concentrations of 80 mM, 180 mM and 275 mM were investigated. Over decades of research indicate multiple mechanisms to withstand soil salinity through biochemical modifications (Parida and Bandhu, 2005). These were aimed to limit salt intake and translocation of ions to the leaves along with osmolyte induction to maintain homeostasis. The results indicate varying coping strategy from declined growth to enhanced root apoplastic barriers in both cultivar Scarlett and wild barley of Pakistan accession. Further transcriptional and physiological attributes also indicate the salt stress specific responses in both barley genotypes.

4.1. Barley root and shoot length are decreased in response to salt stress

Salinity vastly affects the plant growth rate through combined effects of decreased water potential, nutritional imbalance due to the influx of Na ions (Ashraf, 1994; Tester and Davenport, 2003) and accumulation of toxic ions within the cells (Munns *et al*., 1983).

One of the earliest responses was the decrement in the leaf lengths due to the reduction in cell elongation and division (Parida and Bandhu, 2005). After six days of stress treatment, the leaves were much shorter with increasing salt content compared to the control and the development of the second leaf was severely affected, especially with stronger concentrations of 180 mM and 275 mM in both the genotypes. However, the leaves did not visibly undergo any discolouration, even when treated at a maximum concentration of 275 mM which corresponds to a water potential of -1.2 MPa. Other barley studies also exhibited significant decrement in growth under the influence of salinity (Cramer *et al*., 1990; Faralli *et al*., 2015; Wei *et al*., 2003a). The decline in shoot lengths was significant in Scarlett and Pakistan wild barley. The seminal root lengths also varied significantly in response to salinity in both genotypes of barley. Despite Pakistan wild barley having an averagely longer control root; there occurred substantial reduction even at a milder salt concentration of 80 mM where the growth reduced 33.5% in shoot and 30% in roots. Study by Elsawy *et al*., 2018 reported that under salt stress, two Egyptian barley

cultivars (Giza 126 and Giza 128) showed 22.8- 29.7 % shoot reduction and 10.5 – 39.1 % root reduction. Another report by (Ligaba and Katsuhara, 2010) showed that the growth in barley cultivars (K305 and I743) declined up to 40 % when stressed with 100 mM and 50-65 % in 200 mM NaCl. The reduction in both the studies varied differentially according to plant's sensitivity to salt stress and strength of the salinity imposed. On the contrary, it has been also reported that different varieties of barley with varying levels of salt tolerance exhibited a similar reduction in growth rate (Munns *et al*., 1995; Wu *et al*., 2014). In the current study Pakistan wild barley exhibited higher reduction in growth (up to 59.7 %) than in cultivar Scarlett (49.4 %).

Salt stress-response studies had been extensively investigated over the past decades (Chinnusamy *et al*., 2005; G. Cramer *et al*., 1990; Flowers and Hajibagheri, 2001; Hasegawa *et al*., 2000; Munns and Tester, 2008; Zhu, 2002). Comparative physiological effects of salinity and drought stress showed that the former reduced root water uptake and thereby impacted the growth rate, accompanied by hormonal and metabolic level changes very similar to that observed in drought stress (Munns, 2002; Munns *et al*., 1995). In comparison with the previous drought study by Kreszies *et al*. 2019, the average root lengths of Scarlett cultivar stressed with PEG to attain a water potential of -0.4 MPa, -0.8 MPa and -1. 2 MPa were 21.5 ± 4 cm; 19.2 ±6.9 cm and 19.3 ± 3.6 cm respectively; whereas the salt equivalents yielded root lengths of 21.7 ± 3.1cm; 16.7 ±2.6 cm and 12.2 ± 1.4 cm respectively. The greater decrease in the presence of salts was evident. The shoot and root lengths also varied significantly for wild barley Pakistan (Kreszies *et al*., 2020). Further attributes of osmotic stress vs. salt stress will be compared and comprehended in the following sections as well.

The effect of salinity could be sensed by the plants within minutes of salt application (Munns, 2002). Na^+ also disturb the activity of Ca^{2+} which cause a detrimental impact on root growth instantly in cotton crops (Cramer *et al*., 1986). Several studies emphasise the presence of Ca^{2+} in alleviating the decreased growth rate caused by salinity in maize (Cramer *et al*., 1988) and millets (Colmer *et al*., 1996). Ca^{2+} and other elemental variations in salt-stressed leaves and roots will be discussed in further segments.

Other factors influencing the reduced growth rate includes transient turgor loss in leaves (Clipson *et al*., 1985); interference of Na^+ in the apoplast (James *et al*.,2006); modifications in cell wall plasticity (Touchette, 2006). Also, growth decline was exhibited as a measure to overcompensate for the energy expenditure involved in the prevention of salt accumulation, through the synthesis of osmolytes to supplement the nutrient imbalance (Munns *et al*., 2020).

4.2 Casparian bands and suberin lamellae are enhanced in barley roots in response to salt stress

Casparian bands (CB) composed of lignified carbohydrates with aliphatic suberin prevent apoplastic diffusion of solutes. Secondary endodermal differentiation occurred in the form of suberin lamellae (SL) that were arranged radially in the inner tangential cell walls (Schreiber *et al*., 1999). Suberin lamellae comprised of polyphenols and poly-aliphatics (Bernards and Razem, 2001) and the role of suberin as a barrier was attributed to the poly aliphatic domain (Hose *et al*., 2001).

Salt stress of various concentrations had caused complete suberization in endodermal cells at the growing younger part of the roots. It has already been emphasized that salinity stress causes endodermal depositions very closer to the root tip, for example in cotton crop roots (Reinhardt and Rost, 1995). This earlier commence of Casparian band and suberin lamellae formations were histochemically inspected by Fluorol yellow 088 and Aniline-berberine staining respectively (**Figure 6** to **Figure 9**) in both Scarlett and wild barley of Pakistan accession. The development of enhanced suberized barriers commenced within a distance of root tips that contribute 12.5 % of total length in both Scarlett and wild barley from Pakistan during the stress environment.

This formation of complete endodermal suberization in the presence of salts were also observed in previous studies conducted in a variety of plants (Karahara *et al*. 2004; Krishnamurthy *et al*. 2009; Krishnamurthy *et al*. 2011; Reinhardt and Rost 1995; Wang *et al*. 2020). Over the growth period of 12 d in hydroponics, there occurred no development of exodermis in barley not only in salinity stress but also in the course of the drought stress as well (Kreszies *et al*., 2019) for Scarlett and Pakistan accession. During salt stress, the presence of apoplastic barriers is essential to prevent the loading of Na^+ and Cl^- ions into the

xylem (Hasegawa *et al*., 2000) and thereby limiting the transport via symplastic pathway (Nawrath *et al*., 2013; Steudle and Peterson, 1998). This mode of action help in adapting to the Na^+ influx and thereby conferring salt tolerance ability to halophytes such as barley; and also in non-halophytes crops such as rice (Krishnamurthy *et al.*, 2011), cotton (Reinhardt and Rost, 1995) and maize (Karahara *et al*., 2004). In *Arabidopsis thaliana*, aliphatic suberin played an indispensable role in the prevention of solute uptake by the apoplastic pathway and also directed the movement through trans cellular passage (Wang *et al*., 2020). Absence or decreased apoplastic barriers would ensure faster movement of Na ions into the xylem and thereby escalate the accumulation of toxic solutes. These reports critically affirm the role of endodermal suberin barriers in adaptation to the salt stress. The absence of apoplastic barriers in the growing root tips of control roots indicated that the development in stressed roots was induced as a response to salt stress. However, to gain more insight about the transport via apoplast barriers, hydraulic conductivity studies would be helpful as the correlation between endodermal depositions in the form of Casparian bands and suberin lamellae, and hydraulic conductivity had been demonstrated in barley (Knipfer and Fricke, 2011; Kreszies *et al*., 2019, 2020; Ranathunge *et al*., 2017) and in rice (Krishnamurthy *et al*., 2009, 2011; Schreiber *et al*., 2005).

Histochemical analysis of salt-stressed seminal roots of barley showed enhanced suberization starting closer to the root tip (within 25 % of root length). So, to assess the suberin contents and concentration, GC-MS/FID was employed. The concentrations of suberin correspond to endodermal level as the development of exodermis in hydroponically grown barley was not reported (Knipfer and Fricke, 2011; Kreszies *et al*., 2019; Ranathunge *et al*., 2017).

The aliphatic fraction included primary alcohols, fatty acids, di-acids and ω-hydroxy acids (with chain length varying from C_{16} to C_{26}) amongst which $C_{18:1}$ di-acid and C_{18} and C_{24} ω-OHs were found in abundance in both cultivated barley, Scarlett and Pakistan wild barley. The substance classes in the monomer composition deduced in this study were in accordance with the previous works (Kreszies *et al*., 2019; Ranathunge *et al*., 2011; Zeier and Schreiber, 1997). The aromatics consist of aggregates of ferulic and coumaric acids isomers.

The aliphatic suberin concentration in younger root parts, Zone A (0-25 % of root length) varied significantly in response to salt stress ranging between 80 mM and 275 mM in both the genotypes. A maximum fold increase of 6.8 and 5.1 was found in Zone A root segments treated with 275 mM NaCl in Scarlett and wild Pakistan barley respectively. The deposition of suberin in Zone A was expected to be in direct response to the salinity, as the younger part of the roots continued to grow in the stress environment and thereby developing strong apoplastic barriers as an adaptive stress management. In rice also, suberin barriers developed at a distance (1mm), very closer to the root tip (Krishnamurthy *et al*., 2009). These strong suberized barriers could potentially involve in the exclusion of solutes. It had been showed previously in barley that up to 95 % of the salts were excluded from reaching the xylem (Munns, 2002; Munns *et al*., 1983). The role of apoplastic barriers in salt exclusion was further investigated in rice (Miyamoto *et al*., 2001) and it reported that the presence of strongly suberized barriers in response to salinity was crucial in preventing radial water flow. In both the barley genotypes, the concentration of the total aliphatic suberin increased significantly with salt concentrations along the lengths of the root (**Figure 20**). This increment was also reflected in the amount of monomers in the stressed roots. $C_{18:1}$ di-acid and C_{18} and C_{24} ω-OHs were found in the highest aggregates with significant fold changes. The aromatic counterparts also increased along the root length; with significant variations especially at higher stress concentrations of 180 mM and 275 mM NaCl treated roots in both the cultivar and wild type (**Figure 21**).

In Scarlett, the gradient of deposition was linear in stressed roots compared to the control; especially in Zone A, and it appeared that the total aliphatic suberin content between the stress concentrations of 180 mM and 275 mM NaCl did not differ much in Zone B and C. The Pakistan wild barley roots showed the rapid accumulation of aliphatic suberin in Zone A and the amounts were steadily maintained over the lengths of the roots with no sharp increment regardless of the root length and intensity of salt stress. The quantities of total aliphatic suberin in Zone A ranged between 2.3 ± 1.27 $\mu g.cm^{-2}$ and 3.86 ± 1.6 $\mu g.cm^{-2}$ in Scarlett and 3.37 ± 1.12 $\mu g.cm^{-2}$ and 4.94 ± 1.59 $\mu g.cm^{-2}$ in Pakistan wild barley under various salt stress concentrations. But in Zone B and C, the amounts were relatively higher in Scarlett than in the Pakistan variety. In Zone C, Scarlett had a maximum of 9.07 ± 0.69 $\mu g.cm^{-2}$ whereas Pakistan accumulated 6.5 ± 0.69 $\mu g.cm^{-2}$ of total suberin when stressed

with 275 mM NaCl. The differential amounts across the root lengths show rather an accelerated deposition pattern in Pakistan in the younger growing part of the roots which later becomes stagnant over the other zones, than a continuous increment pattern along the root length in Scarlett. From the data, it can be speculated that the wild barley of Pakistan accession tend to suberize at a faster rate than the Scarlett in Zone A. Kreszies *et al*. 2020 showed that during the osmotic stress (-0.8 MPa), seminal roots of wild barley exhibited a delayed suberization than the cultivated barley types. When this data was cross-compared against 180 mM salt stress, which corresponds to the water potential of – 0.8 MPa; an important difference can be observed at the degree of suberization, in Zone A particularly. The amounts of total aliphatic content varied significantly along the lengths of the roots when treated with 180 mM NaCl in both Scarlett and Pakistan barley. On the other hand, during osmotic stress there was no significantly enhanced suberin deposition until 25 % of root length in Scarlett and until 50 % of root length in wild barley of Pakistan accession (Ref data from Kreszies *et al*. 2019, 2020). This distinction in suberin development could be mainly influenced by the presence of salt. Detailed view on apoplastic barriers formed during salt stress and its impact on Na transport in rice was extensively studied by Krishnamurthy *et al*. 2009. The study also showed the variation in Na uptake and accumulation was dependent on the degree of endodermal aliphatic suberin barriers in the roots and confers salinity tolerance to the plants. More discussion about the Na accumulation in shoots will be followed later.

Barley being a halophyte, one of the key mechanisms in adapting to stress and tolerance was through limiting the entry of salt into the root and thereby preventing the build-up of toxic ions within the cells (Munns, 2002). Stronger suberin depositions under salt stress, suggest a significant role of apoplastic barriers in salt tolerance and management in barley. From the quantitative data, the suberin amounts were higher in the young growing part of the Pakistan wild barley than in the Scarlett cultivar.

4.3. Mineral nutrient compositions are affected in barley under salt stress

Among the multiple responses exhibited by plants for salt tolerance, selective accumulation of Na within the shoot and increased compatible solute synthesis (Parida and

Bandhu, 2005) were investigated in this study. Translocation of Na from roots to the shoots is an important physiological mechanism during salt stress (Flowers *et al*., 1977). A previous study suggested that in rice, intracellular compartmentalization of Na within the shoots was a fundamental response to limit Na^+ entry (Anil *et al*., 2005). The results from this study correspond to intracellular Na but whether the accumulation was vacuolar or cytosolic is obscure. Even though both Na and Cl confer toxicity under salt stress environments, the effects of Na have been predominantly studied. Na act as a primary source of ion-induced damage in many crops (Tester and Davenport, 2003) and also disturb ion homeostasis predominantly (Fricke *et al*., 2006). Further investigation of barley salt stress studies by Tavakkoli *et al*. 2011 showed the additive effects of Na and Cl stress responses and that they were independent. And, hence in this study, only the accumulation of Na ions along with the variation of other essential nutrients had been analysed. The results indicated the elevation of Na levels in stressed roots and shoots of Scarlett and Pakistan wild barley. Considering the mild stress concentration of 80 mM, barley genotypes exhibited significant accumulations of Na, thus addressing its halophytic ability to translocate salt to the leaves. The term `osmotic tolerance ´ was coined concerning this trait in halophytes, which primarily dealt with the drought aspect of salt stress (Rajendran *et al*., 2009).

The first obvious question was how the Na from the salt-induced nutrient solution enters and navigates within the plant. Over the years it has been recorded that the transport happened via various channels and transporters including Na^+/H^+ antiporters (Hasegawa *et al*., 2000); xylem loading transporters via cell to cell pathway (Munns, 2002) and radial movement across the root via apoplast pathway (Yeo *et al*., 1987). Na uptake into the shoots of rice was found to be majorly through the apoplastic bypass of the ions (Ranathunge *et al*., 2005).

It was affirmative from the results, that the amounts of Na in leaves had a higher fold increase (Scarlett- 24.1; Pak wild barley- 20.1) than the roots (Scarlett- 7.4; Pak wild barley- 12.1) in both the genotypes. A previous study by Krishnamurthy *et al*., 2011 showed that leaves were more susceptible to Na accumulation and that the transport from root to shoot was majorly unidirectional. Also, halophytes inclined to accumulate Na within the shoots as a mode of tissue tolerance to the salinity (Munns and Tester, 2008; Tester and Davenport, 2003). However, according to Munns 2002, only 2 % of the ions were translocated to the

shoots, citing that the majority would be excluded in the roots. Also, both these processes consume significant amount of energy (Munns *et al*., 2020) and hence investigating the role of apoplastic suberin barriers in preventing Na influx could prove beneficial in conserving energy during the stress.

Another key factor to be considered was the growth system of the plants. Salt stressed rice grown in soil were found to accumulate lesser Na than the ones in hydroponics under the same stress concentrations (Krishnamurthy *et al*., 2009). In the same study, stronger suberized barriers were seen in the soil-grown roots than the hydroponically grown roots; thus linking the Na build-up was inversely proportional to the advent of suberization. In this data, even though the endodermis of Scarlett and Pakistan were completely suberized, significant Na amounts were found in the leaves, thus raising a question about the role of suberin barriers in the uptake and transport of Na to the shoot. This can be explained by the fact that when the plants were transferred after 6 days growth from the control to the nutrient solution containing NaCl immediately Na moved into the root passively against the concentration gradients. Thus Na accumulated within the plant before enhanced apoplastic barriers could be build which is a process of hours (Kreszies *et al*., 2019).

The intrusion of Na into the plant system altered the concentrations of K and Ca significantly .This elemental variation of increased Na accumulation with decreased uptake of K and Ca ions into the shoots was agreeable with previous findings on barley salt stress studies (Tavakkoli *et al*., 2011; Wei *et al*., 2003; Wu *et al*., 2014; Zhu, 2003). It has been emphasised that salt stress cause the root cells to enable the diffusion barriers to limit the water loss and also salt entry (Byrt *et al*., 2018). Also, the binding of ions to the cell wall depends on cation exchange capacity (CEC) and this in turn influence the movement within the system (Marschner, 2011). Na influx through non selective K channels (Rubio *et al*., 2020) or via aquaporins (PIP channels) (Byrt *et al*., 2017) that facilitate the uptake of monovalent cations into the roots. The increased level of salts had been described to induce Ca ion deficiency along with the hindrance of its activity (Cramer *et al*., 1986; Lynch and Lauchli, 1985). Negatively charged cell walls readily binds to cations like Na and Ca and during the salt stress, Na displace Ca ions and eventually inhibit the root elongation (Byrt *et al*., 2018). This effect was reflected on significantly decreased root development in this

study. These responses endorse the deleterious impact of Na on macronutrients that are fundamental for growth and development. From the data, Fe ions concentration increased in stressed conditions and a similar response was observed in Wu *et al*., 2014. It can be seen that the concentration of Fe was higher in roots than the leaves. This could be due to various limitations involved in the translocation of Fe from the root radially through the Casparian strips before xylem loading (Kim and Guerinot, 2007). Also Fe being poorly soluble and highly reactive (Hell and Stephan, 2003) poses additional challenge for long distance transport within the plants. Apoplastic barriers not only limit the Na entry but also restrict the efflux of K and henceforth to maintain high K/Na ratio (Kamiya *et al*., 2015). The results showed up to 20% increase in the levels of P in leaves but not in roots. This could be due to the involvement of mineral elements for osmotic adjustment through enhancement of P content within the leaves (Sima *et al*., 2012), where the Na accumulation fold increase was higher than that in roots in both barley cultivar and wildtype. From the data, there observed no difference between the cultivar Scarlett and wild barley from Pakistan. An earlier investigation of Na content in two barley genotypes (cultivated barley cv. CM72 and Tibetan wild barley XZ16) also showed no significant difference between them (Wu *et al*., 2014). Thus, salinity brought about significant reduction in the amount of Ca and K along with increased accumulation of Na content in both leaf and root tissues. The amounts found in Scarlett and Pakistan wild barley were within similar range and no difference was observed between the genotypes. Also, the Na content in leaves of the stressed plants was greater than that in roots, possibly indicating the translocation of salts from the roots to the shoot.

4.4. Osmotic adjustments to salt stress

Accumulation of Na ions within the cells would eventually lead to osmotic imbalance. The osmotic adjustment occur via increased deposition of low molecular weight organic solutes such as proline (Abraham *et al*., 2003; Hasegawa *et al*., 2000). The amounts of proline increased with the salt concentrations in the current study. This was in accordance with the previous salt stress study on barley by Garthwaite, Von Bothmer, and Colmer 2005 and also in wheat (Poustini *et al*., 2007). Overall, the amounts were higher in Scarlett compared to the wild barley. The leaves had higher amounts than its respective roots in both Scarlett and wild type of Pakistan accession. It can be speculated that higher Na accumulation within the leaves than in the roots could play a contributory effect for

enhanced osmolyte deposition. While there was a substantial increase in leaf Na, the proline levels in 80 mM stressed did not vary significantly from the control. However, at higher concentrations of 180 mM and 275 mM NaCl, there was a significant increment in proline levels. This put forth a question of whether there should be any threshold level of salt concentration for the osmolyte depositions to happen and also for the impact of Na on proline levels and vice versa.

The build-up of proline also occurred in the event of drought stress and in barley, up to a two-fold increase was reported (Kreszies *et al*., 2020). The amounts from this study (180 mM stressed) were within the close range with that of corresponding osmotic stress (-0.8 MPa), and this comparison render a different perspective that the proline accumulation could be initiated due to the osmotic effect induced effect by the salinity. Thus lowering of water potential through salt or PEG might have triggered the reparative accumulation of organic solutes to facilitate osmotic adjustments. Thus, the elevated proline levels in the leaves and roots could be attributed to the Na accumulation and osmotic effect of salt stress respectively.

Another implication of salt stress was observed through changes in osmotic potentials within the roots. Higher the salt concentration, more negative were the measurements and this was in agreement with the previously established results as summarised from several studies by Kumar and Bandhu 2005. The presence of Na ions alongside proline accumulation in roots could have significantly reduced the osmotic potential. In barley, a two-fold decrease in response to osmotic stress (Kreszies *et al*., 2020) compared to 5.6 times reduction in this study, indicate the severity of Na ions in the roots.

4.5. Transcriptomic reprogramming in barley roots as response to salt stress

RNA sequencing for seminal roots (0-12.5 % of length) stressed with 180 mM NaCl was done to gain further insight into the molecular machinery involved in response to salt stress. In this study, the main focus was given to find the differential regulation of genes involved in suberin biosynthesis and transport, salt stress-specific responses, water channels and compatible solute accumulation (proline). GC-MS/FID analysis showed significantly enhanced suberization at the young growing apical part of the root in both Scarlett and

Pakistan accession. In agreement with the quantitative increase, the key genes involved in suberin biosynthesis were positively regulated.

CYP86A1 (cytochrome P450 of CYP86 family), a fatty acid ω-hydroxylase had been identified as a key gene involved in the formation of aliphatic suberin. Reverse genetic studies on Arabidopsis mutants showed a decrement in the concentration of ω-hydroxy acids with chain lengths less than C_{20} and more than 60 % reduction in total aliphatic suberin (Höfer *et al*., 2008). The upregulation of this gene (HORVU3Hr1G085020) was accompanied by significantly elevated amounts of C_{16} and C_{18} ω-OHs in GC analysis in Scarlett. A similar increment in expression was reported in Höfer *et al*. 2008; Li *et al*. 2007. Elaborated studies by Compagnon *et al*., 2009 showed that CYP86B1 (HORVU1Hr1G042810) was necessary for the synthesis of long-chain monomers of ω-OHs and α,ω diacids. Positive log_2FC of CYP86B1 was reflected on analytics where the amounts of C_{22} and C_{24} ω-OHs; also di acids increased significantly. Positive regulation of FAR (Fatty Acyl CoA Reductases) in response to salinity complied with the previous studies (Domergue *et al*., 2010; Vishwanath *et al*., 2013). These studies revealed that FAR1 (HORVU7Hr1G020270) specifically enhances the concentration of C_{22}-ols and FAR 4 (HORVU4Hr1G001450) of C_{20}-ols and the loss of function reduced alcohol content in aliphatic suberin polymer.(Domergue *et al*., 2010; Vishwanath *et al*., 2013). However, the up regulation FAR genes did not vary the alcohols concentration variably in Zone A. BAHD **(**Acyl-CoA dependent acyltransferases), associated with the formation of cell wall polymers such as suberin, lignin (Yu *et al*., 2009) was found to be positively regulated in both Scarlett and Pakistan (HORVU2Hr1G035810). Studies showed that this enzyme functions as ω- hydroxy acid hydroxycinnamoyltransferase and aid in the synthesis of aromatic suberin constituents. The gene induction was evident from increased amounts of aromatic constituents in both varieties. LTP (Lipid transfer proteins) and ABC (ATP binding cassette transporters of G family) were majorly regulated in Pakistan wild type than in Scarlett. Previous works showed that in Arabidopsis, ABCG1 was involved in the transport of long-chained aliphatic suberin monomers to the site of deposition in the roots (Higgins, 2001; Shanmugarajah *et al*., 2019). Contrarily, in the current study, no or weaker gene differentiation was observed. Most of these families of transporters were down-regulated in previous barley salt stress studies as well (Ozturk, 2002). More knowledge on

these transporters could provide much needed clarifications on their expression and mode of function.

Aquaporins are water channels that belong to conserved membrane protein groups and are involved in the movement of water across membranes. Depending on its place of function, the water channels are designated as PIPs (Plasma membrane intrinsic proteins) and TIPs (Tonoplast intrinsic proteins) (Chaumont *et al*., 2001). The regulation of water movement across the channels plays an important role in the adaptation of the plant to stress environments. The data revealed that the aquaporins were prevalently regulated in Pakistan than in Scarlett. Of those that were differentially expressed in Pakistan, most of them were down regulated. In this study, families of PIP2;1 were down-regulated in both the varieties and similar expression was observed by (Katsuhara *et al*., 2002), where three genes corresponding to plasma membrane water channels in barley roots were identified; among which HvPIP 2;1 was predominantly down regulated. The results were relatable to previously published data where PIP1;1 was strongly expressed whilst PIP 2;1 and PIP 2;4 were weakly regulated in response to abiotic stress (Jang *et al*., 2004). In barley roots, most of the aquaporins were not differentially expressed in response to abiotic stress (Coffey *et al*., 2018; Kreszies *et al*., 2019, 2020). Selective expression of genes indicated variability in functional differentiation as proposed previously (Chaumont *et al*., 2000). In aquaporins, differences in the expression could be contributed by a variety of factors including mode of activation sequence, the influence of transcription factors and promoter activity (Di Pietro *et al*., 2013), ubiquitination and post-translational modifications (Casado-Vela *et al*., 2010; D. Y. Kim *et al*., 2013) and so forth. Moreover, it has been emphasized that beneath the genetic expression of aquaporins at the transcriptional level, the regulation also involved additional mechanisms mediated in the form of phosphorylation or dephosphorylation (Maurel *et al*., 2015; Van Wilder *et al*., 2008).

Followed by Na translocation from root to shoot, vacuolar sequestration of Na attributed to osmotic adjustment by preventing the cytosolic Na toxicity in halophytes (Flowers *et al*., 1977) and thereby averting cell damage(Munns, 2002). Ca dependent Salt Overly Sensitive (SOS) pathway model described the inhibition of Na build up within the cytoplasm through a set of proteins- SOS1 (Na^+/H^+ antiporter- NHX1), protein kinase SOS2 (Calcineurin Interacting Protein Kinase- CIPK24) and SOS3 (Calcineurin B Like protein- CBL4)

(Halfter *et al*., 2000). NHX1 studies in barley showed up-regulation in response to salinity (Elsawy *et al*., 2018; Ligaba and Katsuhara, 2010).In the current study, conversely, NHX1 (HORVU2Hr1G021020) was down regulated in wild barley and not differentially expressed in Scarlett. Weaker regulation of NHX1 could increase cytosolic toxicity and in the end, severely impair the growth in barley (Elsawy *et al*., 2018). Though there was a significant growth reduction in 180 mM NaCl treated leaves and roots of both genotypes, there occurred no necrosis or visible damage in the leaves. However, the data presented here were obtained only after six days of stress. Barley being a halophyte, longer stress duration may be needed to incite stronger gene regulation, as comparable salinity studies were done with plants after at least three weeks of stress and growth (Ozturk, 2002; Shi *et al*., 2000). It has been reported that in barley, duration and stress severity increased the number of DEGs (Osthoff *et al*., 2019). CIPK24 (HORVU7Hr1G090260) and CBL4 (HORVU1Hr1G080790) were up regulated in Scarlett and down regulated in Pakistan wild barley roots. This contrast in the expression between the genotypes possibly indicate different mode of expression responses to manage the stress implications.

High-affinity K Transporters (HKT) regulate Na movement to maintain homeostasis and isoforms of HKT1 potentially facilitate Na transport in rice (Horie *et al*., 2001). The results showed that only two forms of HKTs were differentially regulated in Pakistan wild barley. Again, the poor expression could be attributed to several factors including longevity of stress as mentioned earlier and the growth system of barley (Tavakkoli *et al*., 2010). RNA sequencing results showed enhanced expression of the P5CS (Δ1-pyrroline-5-carboxylate synthetase) gene (HORVU1Hr1G072780) in both varieties. This was also reflected as a significant increase when assessed biochemically (**Figure 24**). P5CS was reported to be involved in proline biosynthesis (Delauney and Verma, 1993) and positively influence its accumulation in response to osmotic stress induced by water deficit and salinity (Abraham *et al*., 2003; Dong *et al*., 2010; Khedr *et al*., 2003; Knight *et al*., 1997; Kreszies *et al*., 2020). The role of accumulated osmolytes in the salt tolerance ability was hypothesised to be subjective and dependent on plant species (Ligaba and Katsuhara, 2010).

GO analysis of DEGs showed that in Scarlett, a total of 155 (47 up regulated and 108 down regulated) GO terms were significantly enriched whereas in Pakistan wild barley only 29 (22 up regulated and 7 down regulated) terms were expressed significantly. Salt stress-

induced osmotic effect cause impairment in metabolic activity and thus leading to the formation of Reactive Oxygen Species (ROS) (Cheeseman, 1988; Greenway and Munns, 1980; Halliwell and Gutteridge, 1985). In the current GO study, terms related to catalytic enzymatic reactions such as kinase (GO: 0016301 and GO: 0004672), and transferase (GO: 0016773, GO: 0016740, GO: 0004842, GO: 0019787 and GO: 0016758) were positively regulated. This induction of oxidoreductive enzymes was previously reported in barley roots (Fu *et al*., 2019; Osthoff *et al*., 2019; Ouertani *et al*., 2021). Kumar and Bandhu (2005), summarised efficient handling of ROS was crucial in response to salinity and that their functions contribute to salt tolerance. A recent study from barley root sequencing also suggested that maintenance of osmotic and oxidative homeostasis comply with salt tolerance ability (Ouertani *et al*., 2021). Hence, the enrichment of ROS metabolism specific GO terms in this study indicates the possible response to salt stress management.

Transcription factor activity (GO: 0003700 and GO: 0001071) was enhanced in both the genotypes in this study. In barley during salt stress, transcription factors were reported to be positively regulated according to a previous study by Osthoff *et al*. 2019. Regulation at the transcriptional level had been indicated as a significant factor in salt tolerance (Zhu, 2002). Additionally, enrichment of GO terms related to the modification process (GO: 0036211 GO: 0006464 GO: 004341), protein phosphorylation (GO: 0016310 and GO: 0006468) was observed.

GO terms associated with binding affinity to nucleotide (GO: 0000166), ATP (GO: 0005524), protein (GO: 0005515), carbohydrate (GO: 0097367) and ion (GO: 0043167) were significantly enriched only in Scarlett. This was in accordance with the barley root transcriptome studies by Ouertani *et al*. 2021. Thus, a multitude of mechanisms involving ROS scavenging management, transcription factor activity and other molecular modifications collectively could play a potential role in adaptation and management of the delirious effect imposed by salinity in barley.

The findings from this study contributed only to the transcriptomic responses at 0-12.5 % of total root length. Greater variability in expression can be expected at different root zones (Kreszies *et al*., 2019). Hence, it is important to compare and comprehend differential regulations along the root length as the transcriptional variations within root

zones were higher than that between the treatments within the same species (Hill *et al*., 2016).

4.6. Stomatal conductance decreases in response to salt stress

Salinity affects the important process in plants including photosynthesis and could be mainly due to the osmotic effect (Yeo *et al*., 1985). Stomatal closure was a prominent limiting factor for photosynthesis during salt stress (Chaves *et al*., 2009; Tavakkoli *et al*., 2011). After 30 minutes of stress, a significant reduction compared to control was observed in all three stress concentrations of 80 mM, 180 mM and 275 mM in both genotypes. Nevertheless, the values remained stable over the subsequent days as shown in **Figure 33**. The instantaneous drop in stomatal conductance was also reported in wheat, where the reduction occurred within 45 min after the stress application and the decrement was independent of Na concentration in the leaves (Rahnama *et al*., 2010). This lead to a plausibility that the drop in stomatal conductance could be contributed by the osmotic shock/ stress (Munns and James, 2003; Rahnama *et al*., 2010) and/or as a response due to reduced water potential (Koyro, 2006). The closure of stomata in response to salt stress was also reported to be crucial for tolerance (Robinson *et al*., 1997) and reduction in stomatal conductance was optimal for adaptation to the stress environment to maintain ideal water status (Jones, 1998). The study by Vysotskaya *et al.*, 2010 supported the correlation of lower transpiration rate with enhanced salt tolerance.

The stomatal conductance decreased with increasing salt concentrations and a likewise observation was reported in 10 d salt stressed barley leaves (Yang *et al*., 2009). Maximum fold decrease of 2.7 and 3.7 were observed in Scarlett and Pakistan wild accession respectively in this study, whilst an average of two fold reduction in stomatal conductance was observed in 14 different barley genotypes under salinity (Jiang *et al*., 2006). The study also emphasized stomatal conductance as an integrative parameter to analyse salt stress responses in different barley genotypes. Though there occurred significant reduction due to salt stress, there was no significant difference between Scarlett and Pakistan wild barley and this pattern was in consistence with the study by Kiani-Pouya *et al.*, (2020), where significantly reduced stomatal conductance due to salinity did not differ among the varieties of wild and cultivated barley taken into study.

4.7. Photosynthetic yield and ETR decrement due to salinity

Reduction in stomatal conductance could impact the photosynthesis rate as well (Sharma and Hall, 1991) and was aimed at improving the efficiency of water usage under stress conditions (Osmond *et al*., 2012). Results showed that the photosynthetic yield (Y (II)) and electron transport rate (ETR) reduced with increasing PAR and salt concentrations. Higher intensity of light cause damages in the photosynthetic apparatus, specifically Photosystem II (PSII) (Critchley, 1988). A fluorescence study (Parida and Bandhu, 2005) indicated, while the light intensity affects the PSII, the ability of PS II to recover from the damage was vastly hindered in the presence of salt stress. Elaborative studies reported that salt stress impeded the repair mechanisms of PSII at transcriptional level (Allakhverdiev *et al*., 2002). In both Scarlett and Pakistan varieties, a significant reduction was observed especially in 180 mM and 275 mM stressed leaves. The same effect was reported by Sharma and Hall (1991) in barley, where the combined effect of higher light intensity accompanied by salinity significantly impair the photosynthetic yield, which represents the competence of light reaction (Björkman, 1987). Thus, it can be argued that photoinhibition along with salt stress collectively attribute to the reduction in photosynthetic output in the current study.

Along with the photosynthetic quantum yield, electron transport rate (ETR) also decreased with higher salt stress concentrations comparatively in the present study and were documented in previous studies as well (Basu *et al*., 1998; Parida *et al*., 2003). Unlike stomatal conductance, where significant reduction occurs within 30 minutes of stress application, the photosynthetic yield varied significantly over the elongation of stress period. The importance of stress duration to afflict a notable impact on PS II during salinity had been described previously (Kalaji *et al*., 2011).

Other factors obstructing the activity of PSII and ETR efficiency include, decrease in water potential (osmotic effect) (Allakhverdiev *et al*., 2000; Heuer, 1996), high Na^+/K^+ within the leaves (Ball *et al*., 1987), reduced stomatal conductance (Brugnoli and Björkman, 1992) or other salt specific responses (Greenway and Munns, 1980). It cannot be ignored that the sustainability of photosynthesis rate in saline environments depend vastly on limiting Na accumulation and consequently its build-up within the cytosol (Cramer, 1992; Greenway and Munns, 1980). Thus in this study, the combined effect of high Na accumulation and

decreased stomatal conductance along with strong photoinhibition in leaves negatively afflicted the photosynthetic apparatus evidently reducing the yield and ETR in both Scarlett and Pakistan wild barley. On overall, stressed Pakistan wild barley leaves exhibited greater decrease than the Scarlett leaves, especially at higher salt concentrations of 180 mM and 275 mM (**Figure 34** and **Figure 35**).

4.8. Wax and cutin barriers

Apart from the suberized root barriers, investigations on the leaf barriers were carried out by the GC analysis for wax and cutin in the salt-stressed barley leaves. Hydrophobic barriers in the plant cell walls exist in the form of cuticle and suberin polymers (Graça, 2015; Kolattukudy, 2001; Nawrath, 2002) to facilitate water uptake and movement and also to manage stress environments. The cuticle layer found in the aerial part of the plants comprises polymer matrix cutin; intra-and epicuticular waxes (Müller and Riederer, 2005). Wax and cutin amounts in leaf 1 and leaf 2 of salt-stressed barley were reduced. Class monomers found in GC-MS were in accordance with the previous findings. In both cutin and wax analytics, there was no variation in the amounts in leaf 1, which was almost developed at the time of stress application.

Waxes are typically comprised of very long-chain fatty acids with chain lengths varying up to C_{46}; along with other groups of alcohols, esters, aldehydes and fatty acids (Kolattukudy, 1981; Riederer and Markstädter, 1996). Similar monomer units were found in barley leaves. Total wax concentration did not vary in Scarlett during the stress, but a significant increase was observed in leaf 2 of Pakistan wild barley. However, this increase could be attributed to the severe impairment in the development of leaf 2 when grown in a 180 mM salt-stressed environment; rather than the actual increment of waxes. More information of genetic expression pertaining to wax deposition could provide further clarity. A similar response was discussed in drought-induced cuticular biosynthesis in barley and concluded that cuticular transpiration was not affected despite the change in wax/cutin concentrations (Shellakkutti, 2021).

Increased wax depositions due to salinity in plants had been mainly related to due to the water deficit effect (Shepherd and Griffiths, 2006); and up to 2.5 fold increase had been reported (Kosma and Jenks, 2007). One of the efficient strategies involved in salt tolerance

was through the management of osmotic stress induced by the salinity (Munns *et al*., 2006). As mentioned earlier, the reduced growth rate during salt stress was mainly conferred by the water deficit condition afflicted upon due to salt in the external growth medium. Differential cuticular wax depositions due to salt stress have been reported over the years, where they increased significantly per unit leaf area (Kosma *et al*., 2009); decreased (Hunsche *et al*., 2010) or even remained unchanged (Fricke *et al*., 2006). It cannot be ignored that the variability was possibly influenced by the plant species and salt stress concentrations. This also invites for more cuticular salt stress studies on crops such as barley and rice.

Among the cutin monomers, 9,10 epoxy C_{18} ω-OH was found in most abundance and a similar increase was observed in Espelie *et al*. 1979. C_{16} and C_{18} ω-OHs were commonly found monomers. Total cutin concentrations increased significantly in Scarlett and Pakistan wild barley in leaf 2 of salt-stressed leaves. But when expressed in terms of amount per leaf (µg), no difference was witnessed. The ambiguity in the amounts could be related to the limited development of leaf 2 during the salt stress. Thus it can be subjected to speculation, that the increase was in fact due to the reduced surface area rather than actual induction in response to stress and for the latter to be confirmed, molecular studies would be essential. Studies by Richardson *et al*. 2007 reported that deposition of cutin occur in parallel with leaf elongation while that of wax occur when the elongation terminate in barley. However, more knowledge about the residual transpiration or permeance is needed to further discuss the prospects of wax and cutin depositions during salt stress. Study by Hasanuzzaman *et al*. 2017 reported that reduced water loss because of the cuticular wax deposits subjectively influenced salt tolerance in barley. Hence further study is needed to understand the cuticular behaviour to salinity.

Thus there observed no change in wax and cutin depositions in leaf 1 of both the genotypes. However, the wax amounts in leaf 2 decreased significantly only in Pakistan wild barley and cutin concentrations in leaf 2 of both genotypes decreased significantly. The reduction could be attributed to the poorly developed leaf 2 in the presence of salt stress.

5. SUMMARY

In this study, the responses of 12 d old cultivated barley (*Hordeum vulgare* L. spp.*vulgare)* cv Scarlett and a wild accession (*Hordeum vulgare* spp. *sponataneum*) from Pakistan (ICB181243) to salt stress of concentrations 80 mM, 180 mM and 275 mM corresponding to water potential equivalents of -0.4 MPa, -0.8 MPa and -1.2 MPa were investigated and compared. The shoot and root responses at morphological, histochemical, biochemical, molecular and physiological levels in terms of stress adaptability and tolerance were studied. The salt stress significantly reduced the growth in both species and was prominent in Pakistan wild barley. The suberin amounts from GC showed Pakistan wild barley accumulated greater amounts in the growing root tips (0-25 %) than the Scarlett. However, at 25-100 % of the root lengths, the suberin concentration was higher in Scarlett than in wild species. Pakistan wild barley exhibited rapid suberization rate in younger root parts and the rate was maintained constant over the matured root zones. Scarlett on the other hand, showed linear increase in the suberization pattern along the varying lengths of the root. Thus, a differential pattern in the degree of suberization was observed between the two genotypes.

Further transcriptomic studies in 180 mM stressed roots revealed positive regulation of suberin related genes; and differential regulation of aquaporins and salt stress related genes in both species. The total number of DEGs was higher in wild barley of Pakistan accession than in the Scarlett. GO studies showed the enrichment of terms related to ROS scavenging management, transcription factor activity, ion binding and other molecular modifications that could collectively strategize salt tolerant behaviours in barley. Scarlett cultivar had greater enriched GO terms than the Pakistan wildtype. In conclusion, differential gene analysis showed that wild barley had stronger expression than the cultivar Scarlett; but the gene ontology studies revealed Scarlett had more enriched genes than the wild barley from Pakistan.

Mineral nutrient analysis showed significantly elevated Na accumulation and decreased Ca and K ion levels in both shoots and roots. The leaf tissues had higher fold increase of Na accumulation than the roots. This pattern of Na increase alongside K and Ca

ions decrease was similar in both genotypes. As a consequence of increased Na levels osmotic adjustment through proline occurred in both barley genotypes. Significant increase in proline levels, especially at higher salt stress concentrations of 180 mM and 275 mM was observed in the leaves and roots of both Scarlett and Pakistan wildtype. The overall amounts were higher in Scarlett than in Pakistan barley and this correlates with the stronger expression of proline biosynthesis gene from transcriptomics data. The osmotic potential within the roots reduced significantly than the control. The fold decrease in the stressed roots did not vary much between the genotypes.

The results from physiological studies also conform to the salinity induced effect through reduced stomatal conductance and photosynthetic yield in the leaves. The decrease was more prominent in wild Pakistan barley than in Scarlett. While enhanced apoplastic suberin barriers were observed in the roots; the wax and cutin amounts varied specifically in leaf 2 due to its detrimental development in salinity. The wax and cutin amounts significantly increased in the leaf 2 of Pakistan barley leaves, indicating a stronger response than the Scarlett.

Thus, these results, especially between the cultivar Scarlett and the wild barley from Pakistan show the important role of apoplastic barriers as response to salt stress. The results indicate that wild barley of Pakistan accessions responds more strongly than the cultivar Scarlett in the event of salt stress of different intensities. Further investigations with more wild barley genotypes will improve our understanding of salt stress response and tolerance processes and can be used in future breeding programs to gain more salt tolerant crops.

6. SUPPLEMENTARY DATA

Table S1 - Cross comparison of enriched GO terms in Up regulated DEGs in the roots of Scarlett and Pakistan wild barley.

Only GO terms with FDR <0.05 were considered. GO- Gene Ontology; Onto- Ontology; P- Biological Process; F- Molecular Function; C- Cellular Component ; FDR- False Discovery Rate.

			GO Information	Stress vs.Control		Pak_Svs.C		Sca_Svs.C	
No	GO Term	Onto	Description	Pak	Sca	FDR	Num	FDR	Num
1	GO:0036211	P	protein modification process			0.0023	115	1.6e-05	249
2	GO:0006464	P	cellular protein modification process			0.0023	115	1.6e-05	249
3	GO:0043412	P	macromolecule modification			0.008	115	9,00E-05	251
4	GO:0016310	P	phosphorylation			0.0096	100	2.9e-05	223
5	GO:0006468	P	protein phosphorylation			0.01	93	1.6e-05	212
6	GO:0007264	P	small GTPase mediated signal transduction			0.047	13	---	---
7	GO:0003824	F	catalytic activity			2.9e-06	451	0.0046	939
8	GO:0016740	F	transferase activity			3.2e-05	197	4.3e-07	424
9	GO:0016757	F	transferase activity, transferring glycosyl groups			0.0005	49	---	---
10	GO:0016301	F	kinase activity			0.004	102	3.2e-07	238
11	GO:0016773	F	phosphotransferase activity, alcohol group as acceptor			0.004	101	3.2e-07	236
12	GO:0004672	F	protein kinase activity			0.0043	94	1.8e-06	215
13	GO:0016772	F	transferase activity, transferring phosphorus-containing groups			0.016	109	8.9e-07	260
14	GO:0016758	F	transferase activity, transferring hexosyl groups			0.019	35	---	---
15	GO:0005509	F	calcium ion binding			0.02	28	---	---
16	GO:0015018	F	galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase activity			0.026	5	---	---
17	GO:0015020	F	glucuronosyltransferase activity			0.026	5	---	---
18	GO:0004842	F	ubiquitin-protein transferase activity			0.037	14	0.045	24
19	GO:0019787	F	ubiquitin-like protein transferase activity			0.037	14	0.045	24
20	GO:0004553	F	hydrolase activity, hydrolyzing O-glycosyl compounds			0.046	34	---	---
21	GO:0001071	F	nucleic acid binding transcription factor activity			0.046	36	0.012	74
22	GO:0003700	F	transcription factor activity, sequence-specific DNA binding			0.046	36	0.012	74
23	GO:0006796	P	phosphate-containing compound metabolic process			---	---	0.00086	247
24	GO:0006793	P	phosphorus metabolic process			---	---	0.00088	247
25	GO:0097659	P	nucleic acid-templated transcription			---	---	0.014	149
26	GO:0006351	P	transcription, DNA-templated			---	---	0.014	149
27	GO:0032774	P	RNA biosynthetic process			---	---	0.014	149
28	GO:2001141	P	regulation of RNA biosynthetic process			---	---	0.027	127
29	GO:0006355	P	regulation of transcription, DNA-templated			---	---	0.027	127
30	GO:1903506	P	regulation of nucleic acid-templated transcription			---	---	0.027	127
31	GO:0051252	P	regulation of RNA metabolic process			---	---	0.027	127
32	GO:0010468	P	regulation of gene expression			---	---	0.042	130
33	GO:0019219	P	regulation of nucleobase-containing compound metabolic process			---	---	0.042	127

34	GO:0005488	F	binding			---	---	0.001	1263
35	GO:0032559	F	adenyl ribonucleotide binding			---	---	0.001	336
36	GO:0005524	F	ATP binding			---	---	0.001	296
37	GO:0030554	F	adenyl nucleotide binding			---	---	0.001	336
38	GO:0043565	F	sequence-specific DNA binding			---	---	0.0014	56
39	GO:0032549	F	ribonucleoside binding			---	---	0.0061	349
40	GO:0017076	F	purine nucleotide binding			---	---	0.0061	350
41	GO:0032555	F	purine ribonucleotide binding			---	---	0.0061	349
42	GO:0032550	F	purine ribonucleoside binding			---	---	0.0061	349
43	GO:0001883	F	purine nucleoside binding			---	---	0.0061	349
44	GO:0001882	F	nucleoside binding			---	---	0.0061	349
45	GO:0035639	F	purine ribonucleoside triphosphate binding			---	---	0.0061	309
46	GO:0000166	F	nucleotide binding			---	---	0.0072	412
47	GO:1901265	F	nucleoside phosphate binding			---	---	0.0072	412
48	GO:0032553	F	ribonucleotide binding			---	---	0.0072	352
49	GO:0005515	F	protein binding			---	---	0.0072	484
50	GO:0097367	F	carbohydrate derivative binding			---	---	0.0072	354
51	GO:0043169	F	cation binding			---	---	0.0072	318
52	GO:0036094	F	small molecule binding			---	---	0.0079	414
53	GO:0043167	F	ion binding			---	---	0.0092	332
54	GO:0046872	F	metal ion binding			---	---	0.013	309

Table S2 - Cross comparison of enriched GO terms in Down regulated DEGs in the roots of Scarlett and Pakistan wild barley.

Only GO terms with FDR <0.05 were considered. GO- Gene Ontology; Onto- Ontology; P- Biological Process; F- Molecular Function; C- Cellular Component ; FDR- False Discovery Rate.

			GO Information	Stress vs.Control		Pak_Svs.C		Sca_Svs.C	
No	GO Term	Onto	Description	Pak	Sca	FDR	Num	FDR	Num
1	GO:0005515	F	protein binding			4.2e-06	525	---	---
	GO:0003690	F	double-stranded DNA binding			0.0026	21	---	---
3	GO:0005488	F	binding			0.042	1244	---	---
4	GO:0008270	F	zinc ion binding			0.042	152	---	---
5	GO:0043227	C	membrane-bounded organelle			0.0065	145	9.6e-11	88
6	GO:0043231	C	intracellular membrane-bounded organelle			0.0065	145	9.6e-11	88
7	GO:0015630	C	microtubule cytoskeleton			0.011	13	---	---
8	GO:0006518	P	peptide metabolic process			---	---	6.9e-27	112
9	GO:0043043	P	peptide biosynthetic process			---	---	7.6e-27	110
10	GO:0043604	P	amide biosynthetic process			---	---	7.6e-27	110
11	GO:0043603	P	cellular amide metabolic process			---	---	7.6e-27	112
12	GO:0006412	P	translation			---	---	1.2e-26	109
13	GO:1901566	P	organonitrogen compound biosynthetic process			---	---	2,00E-26	133

14	GO:1901564	P	organonitrogen compound metabolic process			---	---	3.7e-26	152
15	GO:0006807	P	nitrogen compound metabolic process			---	---	2.1e-14	210
16	GO:0034645	P	cellular macromolecule biosynthetic process			---	---	1.5e-13	156
17	GO:0009059	P	macromolecule biosynthetic process			---	---	1.5e-13	156
18	GO:0034641	P	cellular nitrogen compound metabolic process			---	---	6.1e-13	192
19	GO:0006333	P	chromatin assembly or disassembly			---	---	6.1e-13	25
20	GO:0044249	P	cellular biosynthetic process			---	---	6.4e-13	185
21	GO:1901576	P	organic substance biosynthetic process			---	---	6.5e-13	185
22	GO:0009058	P	biosynthetic process			---	---	1.6e-12	190
23	GO:0034728	P	nucleosome organization			---	---	1.9e-12	24
24	GO:0031497	P	chromatin assembly			---	---	1.9e-12	24
25	GO:0006334	P	nucleosome assembly			---	---	1.9e-12	24
26	GO:0044271	P	cellular nitrogen compound biosynthetic process			---	---	1.9e-12	153
27	GO:0065004	P	protein-DNA complex assembly			---	---	2,00E-12	24
28	GO:0071824	P	protein-DNA complex subunit organization			---	---	2,00E-12	24
29	GO:0006323	P	DNA packaging			---	---	3.1e-12	24
30	GO:0010467	P	gene expression			---	---	6.9e-12	151
31	GO:0071103	P	DNA conformation change			---	---	9.1e-12	25
32	GO:0044085	P	cellular component biogenesis			---	---	1.3e-10	43
33	GO:0006325	P	chromatin organization			---	---	8.4e-10	26
34	GO:0070271	P	protein complex biogenesis			---	---	5.3e-09	27
35	GO:0006461	P	protein complex assembly			---	---	5.3e-09	27
36	GO:0034622	P	cellular macromolecular complex assembly			---	---	5.8e-09	27
37	GO:0009987	P	cellular process			---	---	8.9e-09	344
38	GO:0071822	P	protein complex subunit organization			---	---	1.1e-08	28
39	GO:0071840	P	cellular component organization or biogenesis			---	---	1.1e-08	57
40	GO:0065003	P	macromolecular complex assembly			---	---	1.4e-08	27
41	GO:0051276	P	chromosome organization			---	---	3.6e-07	27
42	GO:0043933	P	macromolecular complex subunit organization			---	---	3.6e-07	30
43	GO:0006996	P	organelle organization			---	---	5.8e-07	32
44	GO:0022607	P	cellular component assembly			---	---	2.3e-06	27
45	GO:0016043	P	cellular component organization			---	---	6.8e-05	41
46	GO:0006839	P	mitochondrial transport			---	---	0.0019	6
47	GO:0044267	P	cellular protein metabolic process			---	---	0.0019	140
48	GO:0007264	P	small GTPase mediated signal transduction			---	---	0.0031	13
49	GO:0015031	P	protein transport			---	---	0.0041	22
50	GO:0045184	P	establishment of protein localization			---	---	0.0055	22
51	GO:0044237	P	cellular metabolic process			---	---	0.0059	267
52	GO:0008104	P	protein localization			---	---	0.0062	22
53	GO:0010410	P	hemicellulose metabolic process			---	---	0.007	8
54	GO:0010383	P	cell wall polysaccharide metabolic process			---	---	0.007	8
55	GO:0044260	P	cellular macromolecule metabolic process			---	---	0.0084	206
56	GO:1901605	P	alpha-amino acid metabolic process			---	---	0.01	13
57	GO:0071702	P	organic substance transport			---	---	0.013	28
58	GO:0046907	P	intracellular transport			---	---	0.013	18
59	GO:0051649	P	establishment of localization in cell			---	---	0.013	18

60	GO:0044036	P	cell wall macromolecule metabolic process			---	---	0.015	9
61	GO:0051641	P	cellular localization			---	---	0.021	18
62	GO:0009067	P	aspartate family amino acid biosynthetic process			---	---	0.032	6
63	GO:0006886	P	intracellular protein transport			---	---	0.036	16
64	GO:0009066	P	aspartate family amino acid metabolic process			---	---	0.036	6
65	GO:0051169	P	nuclear transport			---	---	0.044	7
66	GO:0006913	P	nucleocytoplasmic transport			---	---	0.044	7
67	GO:0042546	P	cell wall biogenesis			---	---	0.045	9
68	GO:0033036	P	macromolecule localization			---	---	0.048	22
69	GO:0005198	F	structural molecule activity			---	---	7.8e-32	107
70	GO:0003735	F	structural constituent of ribosome			---	---	2.9e-31	103
71	GO:0046982	F	protein heterodimerization activity			---	---	3.3e-30	64
72	GO:0046983	F	protein dimerization activity			---	---	1,00E-20	77
73	GO:0003677	F	DNA binding			---	---	0.0041	100
74	GO:0003676	F	nucleic acid binding			---	---	0.025	144
75	GO:0043232	C	intracellular non-membrane-bounded organelle			---	---	1.7e-63	174
76	GO:0043228	C	non-membrane-bounded organelle			---	---	1.7e-63	174
77	GO:0043229	C	intracellular organelle			---	---	8.5e-53	223
78	GO:0043226	C	organelle			---	---	8.5e-53	223
79	GO:0044464	C	cell part			---	---	1.2e-51	277
80	GO:0005623	C	cell			---	---	1.2e-51	277
81	GO:0005622	C	intracellular			---	---	5.4e-50	268
82	GO:0032991	C	macromolecular complex			---	---	5.4e-50	199
83	GO:0044424	C	intracellular part			---	---	5.1e-46	250
84	GO:1990904	C	ribonucleoprotein complex			---	---	2.7e-34	110
85	GO:0030529	C	intracellular ribonucleoprotein complex			---	---	2.7e-34	110
86	GO:0000786	C	nucleosome			---	---	1.8e-32	63
87	GO:0044815	C	DNA packaging complex			---	---	1.9e-32	63
88	GO:0032993	C	protein-DNA complex			---	---	1.9e-32	63
89	GO:0000785	C	chromatin			---	---	2.8e-32	63
90	GO:0005737	C	cytoplasm			---	---	3,00E-32	150
91	GO:0044444	C	cytoplasmic part			---	---	5.1e-32	134
92	GO:0005840	C	ribosome			---	---	5.1e-32	102
93	GO:0044422	C	organelle part			---	---	4,00E-31	108
94	GO:0044446	C	intracellular organelle part			---	---	4,00E-31	108
95	GO:0044427	C	chromosomal part			---	---	9.4e-31	63
96	GO:0005694	C	chromosome			---	---	4.1e-30	64
97	GO:0043234	C	protein complex			---	---	3.1e-15	89
98	GO:0005634	C	nucleus			---	---	1,00E-07	62
99	GO:0031975	C	envelope			---	---	0.00054	13
100	GO:0005740	C	mitochondrial envelope			---	---	0.00058	12
101	GO:0044429	C	mitochondrial part			---	---	0.00075	13
102	GO:0031966	C	mitochondrial membrane			---	---	0.001	11
103	GO:0005739	C	mitochondrion			---	---	0.0014	17
104	GO:0031090	C	organelle membrane			---	---	0.0016	14
105	GO:0031967	C	organelle envelope			---	---	0.0016	12

106	GO:0098798	C	mitochondrial protein complex			---	---	0.0019	6
107	GO:0019867	C	outer membrane			---	---	0.0062	6
108	GO:0044455	C	mitochondrial membrane part			---	---	0.0071	6
109	GO:0005741	C	mitochondrial outer membrane			---	---	0.0082	5
110	GO:0031968	C	organelle outer membrane			---	---	0.0098	5
111	GO:0098588	C	bounding membrane of organelle			---	---	0.023	8
112	GO:0098805	C	whole membrane			---	---	0.023	7
113	GO:0044391	C	ribosomal subunit			---	---	0.041	13

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