Effect of Salt Stress on Growth, Biochemical and Molecular Changes in Local Rice Plant (*Oryza sativa* L.)

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DECLARATION

I, **Mrs. Smita Srivastava,** hereby declare that this thesis represents work which has been carried out by me and that it has not been submitted, either in part or full, to any other University or Institution for the award of any research degree.

Place: Taleigao Plateau. **Mrs. Smita Srivastava**

Date: 06-01-2023

CERTIFICATE

I hereby certify that the above declaration of the candidate, **Mrs. Smita Srivastava** is true, and the work was carried out under my supervision.

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Mrs. Smita Srivastava

This thesis is dedicated to

my Family

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$^1O_2$	Singlet oxygen
AA/DHA	Ascorbate - dehydroascorbate
AA-GSH	Ascorbate- Glutathione cycle
ABPs	Actin-binding proteins
APX	Ascorbate peroxidase
AsA	Ascorbic acid
ATP	Adenosine triphosphate
AtPCS	*Arabidopsis thaliana* phytochelatin synthase
BjGLYI	*B. juncea*, Glyoxalase I
CAT	Catalase
CEC	Cation exchange capacity
Chl a	Chlorophyll ‘a’
Chl b	Chlorophyll ‘b’
Chl*	Excited chlorophyll molecule
CLC	Chloride channel
CP12	Calvin cycle protein
cPGK,	Cytosolic phosphoglycerate kinase
Cu/Zn-SOD	Copper/Zinc Super Oxide Dismutase
Cyt b6/f	Cytochrome b6/f
cyt f	Cytochrome f
DHAR	Dehydroascorbate reductase
dS m^{-1}	DeciSiemens per meter
DW	Dry weight
ECe	Electrical conductivity of the element
eIF	Eukaryotic translation initiation factor
EL	Electrolyte leakage
ESP	Exchangeable sodium percentage
ETR	Electron transport rate
FAD	Flavin adenine dinucleotide
Fd	Ferredoxin
Fe-SOD	Iron-SOD
IDA	Information-dependent acquisition
Fm	Maximum fluorescence
FMS	Fluorescence monitoring system

Fv	Variable fluorescence
FW	Fresh weight
G6PI	Glucose-6-phosphate isomerase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC-MS	Gas chromatography-mass spectrometry
GLY I	Glyoxalase I
GPX	Guaiacol peroxidase
GR	Glutathione Reductase
GRPs	Glycine rice RNA binding proteins
GS	Glutamine synthetase
GSH/GSSG	Glutathione/Oxidized form of glutathione
GST	Glutathione S-Transferases
H_2O_2	Hydrogen peroxide
HKT1	High-affinity potassium transporter gene
HPLC	High-pressure liquid chromatography
HSPs	Heat shock proteins
HvHKT2;1	Hordeum vulgare, K^+ transporter
IRGA	Infra-RED Gas Analyzer
LC-MS	Liquid chromatography-Mass spectrometry
LHC	Light harvesting complex
LOX	Lipooxygenase
LPO	Lipid peroxidation
LTPs	Lipid transfer proteins
MDA	Malondialdehyde
MDH	Malate dehydrogenase
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
MG	Methylglyoxal
Mg-chl	Magnesium - chlorophyll
MGDG	Monogalactosyldiacylglycerol
Mn-SOD	Manganese-SOD
NADPH/NADP	Nicotinamidediphosphate hydrogen/Nicotinamidediphosphate
NHX1	Tonoplast-localized Na^+/H^+ exchanger
NtAQP1	Tobacco membrane-intrinsic proteins
OEC	Oxygen evolving complex

OEE 1	Oxygen evolving enhancer
OH·	Hydroxyl radical
OsHKT1;1	Sodium Transporter
OsHKT2;4	Na^+/K^+ is a co-transporter
OsP5CS	*Oryza sativa* Proline Synthesis gene
PCD	Programmed cell death
PG 3-	Phosphoglycerate
PGK	Phosphoglyceric acid kinase
PGK	Phosphoglycerate kinase
PGM	Phosphoglycerate mutase
PIPs	Plasma membrane intrinsic proteins
PK	Pyruvate kinase
POD	Peroxidase
PSI	Photosystem I
PSII	Photosystem II
PUFA	Polyunsaturated fatty acids
PUFAs	Polyunsaturated fatty acids
qNP	Non-photochemical quenching
qP	Photochemical quenching
RBP	Rubisco binding protein
RC	Reaction centers
RMtATP6	ATP synthase small subunit gene
ROO·	Lipid peroxy radical
ROOH	Lipid hydroperoxide
ROS	Reactive Oxygen Species
RT-PCR	Real-Time Polymerase Chain reaction
RubisCO	Ribulose bisphosphate carboxylase/oxygenase
RWC	Relative water content
SAR	Sodium adsorption ratio
SEM	Scanning Electron Microscope
SOD	Superoxide dismutase
SOS1	Plasma membrane-localized Salt Overly Sensitive 1
SWATH	Sequential window acquisition of all theoretical fragment ion spectra
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances

TCA cycle	A tricarboxylic cycle
TPI	Triosephosphate isomerase
USD	United states dollar
WUE	Water use efficiency
Ψ	Water potential
ψg	Gravitational potential
ψm	Matric potential
ψp	Pressure potential
ψs	Osmotic potential
Ψl	Leaf water potential

ABSTRACT

Salt stress in the coastal saline soil of Goa is a serious detriment in rice productivity (*Oryza sativa* L.). This study compared the morphological, physio-biochemical, and molecular responses of indigenous salt-tolerant 'Korgut' and salt-sensitive 'Jaya' rice varieties by pot culture in controlled climatic conditions. Decreased plant growth and biomass with the increase in salinity were recorded in the 'Jaya' variety compared to those of 'Korgut'. Salt tolerance of the 'Korgut' variety depended on their ability to maintain better relative water content (RWC) than 'Jaya'. A significant decline was observed in net photosynthesis rate (P_N), transpiration rate (E), stomatal conductance (gs), internal CO_2 concentration (Ci), photochemical quenching, and lower quantum efficiency of the PSII system (Fv/Fm ratio) in 'Jaya' compared to those of 'Korgut' as a response to salinity stress. Similarly, photochemical quenching (qP) significantly decreased in 'Jaya' compared to those of 'Korgut,' which displayed an enhanced level of non-photochemical quenching (qNP) compared to the 'Jaya' variety. A significant decline in chlorophyll content was observed in 'Jaya' than in 'Korgut'. Increased trichomes number and size and reduction in the stomatal size due to salt treatment in 'Korgut' indicated the role of morphological adaptation to salt tolerance by preventing water loss. The chloroplast ultrastructure of the salt-sensitive 'Jaya' was more severely affected by NaCl stress than the salt-tolerant 'Korgut' variety. The tolerance of 'Korgut' was mainly observed due to a relatively lesser accumulation of Na^+ and Cl^- and a higher accumulation of K^+ compared to those of the 'Jaya' rice variety under salinity stress. These observations were further substantiated by gene expression of OsHKT1;1 (Na^+ specific transporter) and OsHKT2;4 (a K^+/Na^+ symporter) as the expression level suggested better osmoregulation and ion homeostasis in the 'Korgut' variety. 'Jaya' variety of rice exhibited a greater amount of oxidative damage, electrolyte leakage (EC), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•), lipid peroxidation (MDA), and protein carbonyl (CO) production compared to those of 'Korgut' rice variety under salinity stress. 'Korgut' showed relatively higher antioxidant capacity (SOD, APX, and CAT) and lower osmotic stress (lower proline) compared to the salt-sensitive variety 'Jaya'. These results were further substantiated by greater expression of SOD and APX in 'Korgut' than in 'Jaya,' suggesting better protection against oxidative damage. An increased level of OsP5CS1 expression (proline biosynthesis gene) indicated physiological drought as the result of salt stress. GC-MS analysis showed higher levels of saturated fatty acids (palmitic acid and stearic acid) in 'Jaya' than in the 'Korgut' variety.

In contrast, the ‘Korgut’ variety displayed an enhanced level of unsaturated fatty acid (oleic acid, linoleic acid, and α-linolenic acid), which may suggest an adaptational role probably through maintaining membrane fluidity in making ‘Korgut’ more tolerant to salt stress. Thylakoid membrane protein, studied using SDS-PAGE, also decreased after NaCl treatment, which is more prominent in the ‘Jaya’ than the ‘Korgut’ variety. Photo-inactivation of PSII in ‘Jaya’ includes the loss of the D1 and D2 (32-34kDa) protein, probably due to greater photosynthetic damage caused by salinity stress. In ‘Jaya’, the most noticeable decline of the 47kDa chlorophyll protein (CP), 17kDa, and (23 &10kDa) OEC protein suggest decreased energy transfer from the light-harvesting antenna to PSII due to the marked alterations in the composition of thylakoid membrane proteins. Proteomics data analyzed using LC-MS/MS revealed more than 2-fold upregulation of 86 proteins in ‘Korgut’ with the highest salt concentration. Increased proteins in ‘Korgut’ were related to the photosynthetic, glycolytic, and pathway, salt-responsive proteins, and protein biosynthetic processes. In addition, up-regulation of lipid transfer proteins, actin-binding proteins, and PIP1 related to aquaporin also showed up-regulation in ‘Korgut’. ‘Jaya’ also showed a less than 2-fold upregulation of 46 proteins. Our study concluded that the ‘Korgut’ variety maintained ion and water homeostasis and a higher threshold level of antioxidant enzymes, which limits ROS-mediated oxidative damage and upregulates the processes associated with the higher photosynthesis that enhanced the biomass, overcoming salinity stress and enabling sustainable growth.

CHAPTER 1

INTRODUCTION

“To be a successful farmer, one must first know the nature of the soil.”

Xenophon, Oeconomicus,

1.1 Salinity

With a burgeoning population estimated to reach around 1.43 billion by 2030, India requires approximately 311 million tons of cereals and pulses to achieve food security. Further, there is a need to increase food grain production to 350 million tons to feed the estimated population of 1.8 billion by 2050 (Kumar & Sharma, 2020). To meet the future food security target, it is expected to increase food grain production by 2 million tons per annum. To increase food grain production, there is a need to expand agricultural land and crop productivity. Contrarily rapid urbanization and industrialization have led to the shrinkage of agricultural land. In India, as reported in 2015, nearly 147 million ha of land undergoes soil degradation, with 94 million ha due to water erosion, 23 million ha due to salinity, alkalinity, or acidification, 14 million ha from water-logging, 9 million ha from wind erosion, and 7 million ha from a combination of factors (Bhattacharyya et al., 2015). In India, 6.74 million ha of land is salt-affected, and with an annual 10% increase, soil salinization is projected to cover 16.2 million ha by 2050 (Sharma, 2015). To utilize the salinized land, development of crops that can tolerate salinity is needed. Decades of research by the Indian Council of Agricultural Research (ICAR), and several agricultural universities have highlighted the need to understand the causes of salt accumulation and plant responses to salt stress to adopt suitable technologies for the management of salt-affected soils. Crops that tolerate high salinity levels in the soil would be a practical contribution toward addressing the problem. Most crops tolerate salinity to a threshold level ($\geq 4 dsm^{-1}$) below which crop growth is not affected.

According to the FAO report, salt-affected lands exist in more than 100 countries globally, with more than 831 million ha (Martinez-Beltran, 2005). Salinity is causing massive annual economic losses worldwide of over 10 billion USD. This amount exceeds the gross domestic product of more than 50 less-developed countries in the modern world (Qadir et al., 2014).

Salt stress is estimated to increase tremendously in future climate change scenarios due to sea-level rise and its effect on coastal areas. The temperature rise will inevitably lead to increased evaporation and further salinization. Some well-known regions where salinization is extensively reported include the Aral Sea Basin (Amu-Darya and Syr-Darya River Basins) in Central Asia and the Indo-Gangetic Basin in India. The countries affected by salinization are majorly located in arid and semi-arid regions, where low-quality groundwater is used for continuous irrigation (Massoud, 1974; Ponnamperuma, 1984). The green land is converted to barren land rapidly due to the salinization of land.

Worldwide, around 10 million hectares of irrigated land are abandoned because of salinization, sodication, and waterlogging (Szabolcs, 1989). These degraded soils occur mainly in the hot arid, and semi-arid regions, although it has also been recorded in Polar Regions (Buringh & Buringh, 1979), Basin in Pakistan, the Euphrates Basin in Syria and Iraq, the Yellow River Basin in China, the Murray-Darling Basin in Australia, and the San Joaquin Valley in the United States (Qadir et al., 2014). In Asia, 20% of India's cultivable land is affected by salinity or sodicity, mainly in Rajasthan, coastal Gujarat, and the Indo-Gangetic plain. Shahid et al. (2018) have reported the extent of saline to be 10×10^6 ha in Pakistan, 3×10^6 hectares in Bangladesh, 3.58×10^6 hectares in Thailand, 26×10^6 ha in China, and 357×10^6 ha in Australia. According to the NRSA/DOS project on 'Mapping of salt-affected soils of India on 1:250,000 scale', the area under salt-affected soils constitutes around 6.727 Mha (million hectare), out of which coastal saline soils account for 3.1 million ha, and the remaining 3.771 Mha is sodic (Arora & Sharma, 2017) (Table 1.1).

Salinity causes a significant loss in crop yield in temperate and tropical climates (Ayyogari et al., 2014). It adversely affects plant metabolism and physiological functions such as growth, photosynthesis, protein synthesis, enzyme activity, membrane disorganization, energy, and lipid metabolism in multiple ways due to the induction of osmotic and oxidative stress and ion/salt toxicity. Data from 2012-2014 suggests that due to soil salinization, India annually loses crop production of nearly 16.84 million tons, valued at Rs 230 billion (Mandal et al., 2018). An increase in the amount of salt in the soil causes osmotic stress. This decreases the amount of water that the plant uses, resulting in a physiological drought leading to ionic stress. Continuous exposure leads to ion toxicity (salt stress) or hyperionic stress. Under salt stress, Na^+ and Cl^- ion concentration is increased, reducing essential nutrients such as K^+, Ca^{2+}, and Mg^{2+} and causing nutrient deficiency in the plant (Yildiz et al., 2020). A low molecular mass compound known as compatible solute is accumulated under salt stress to provide solute potential to maintain water potential in favor of plant nutritional disorders, germination, and plant vigor, leading to crop productivity loss (Fig. 1.1).

Table 1.1: Distribution of Saline/Sodic Soils in India (%), adopted from Arora and Sharma (2017).

Sr. No.	State	Sodic soils	Saline soils	Costal saline soil	Total
1	Gujarat	14.3	71.2	37.1	32.9
2	Uttar Pradesh	35.6	1.3	-	20.3
3	Maharashtra	11.2	10.4	0.6	9.0
4	West Bengal	-	-	35.4	6.5
5	Rajasthan	4.7	11.4	-	5.6
6	Tamil Naidu	9.4	-	1.1	5.5
7	Andhra Pradesh	5.2	-	6.2	4.1
8	Haryana	4.8	2.9	-	3.4
9	Bihar	2.8	2.8	-	2.3
10	Punjab	4.0	-	-	2.2
11	Karnataka	3.9	0.1	-	2.2
12	Orissa	-	0	11.8	2.2
13	Madhya Pradesh	3.7	-	-	2.1
14	Andaman and Nicobar Island	-	0	6.2	1.1
15	Kerala	-	0	1.6	0.3
	Total	100 (3.78Mha)	100 (1.71Mha)	100 (1.21Mha)	100 (6.47Mha)

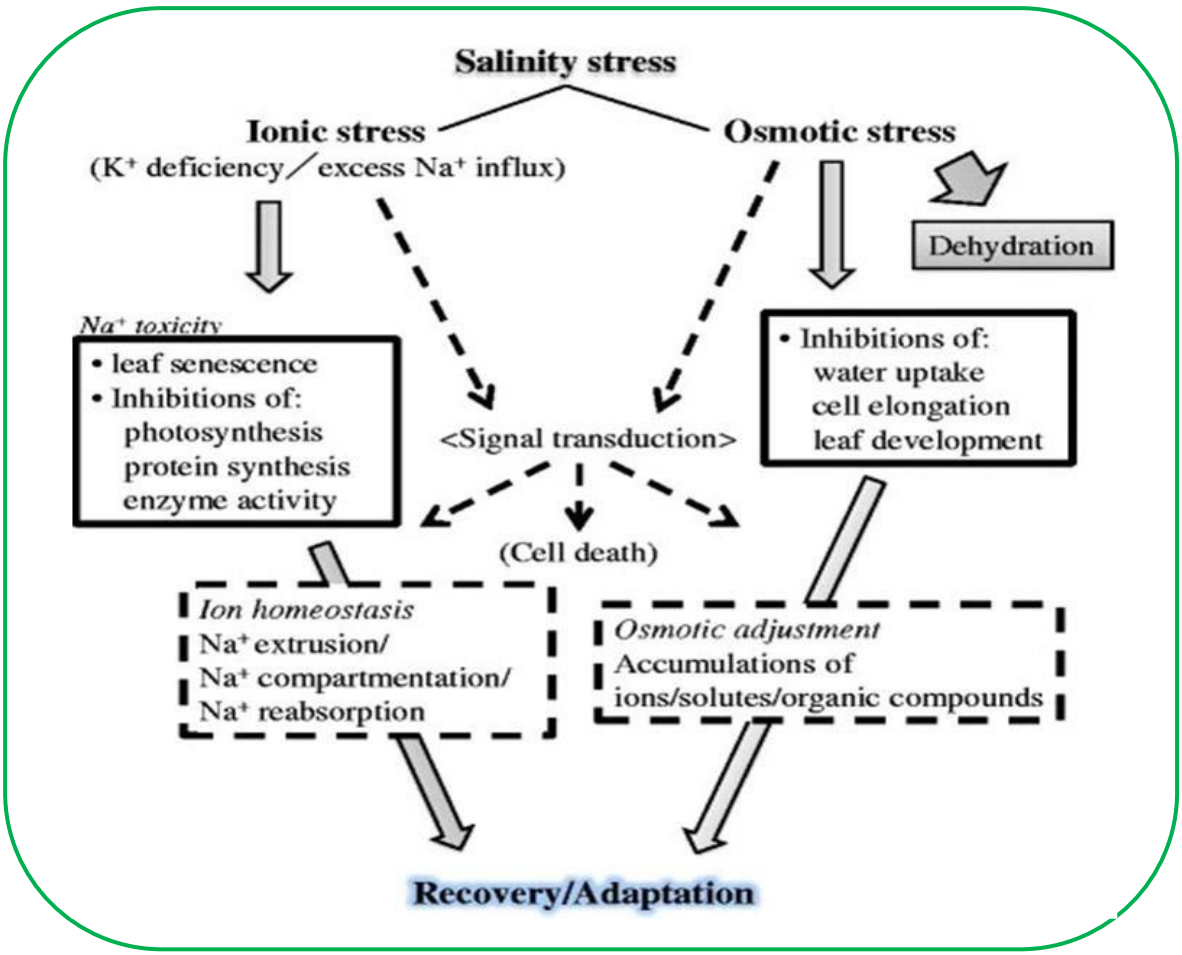


Fig. 1.1 A schematic summary of plant stresses under high salinity conditions and the corresponding responses that plants use to survive these detrimental effects (Horie et al., 2012).

1.1.1 Causes and types of salinity

Salinity refers to salts such as sodium chloride, magnesium and calcium sulfates, and bicarbonates in soil and water. Salinity can occur naturally, referred to as primary salinity, such as rocks containing salts of chlorides of sodium, calcium, and magnesium, and to a lesser extent sulfates and carbonates. Seawater and brackish water are other sources of salinity in low-lying coastal regions. Irrigation is the leading cause of salinity in most cultivated land during the high tide of seawater which causes a rise in the water table and an accumulation of salts in the root zone. Post-cultivation salts such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), often precipitate into carbonates leading to the accumulation of Na^{+} in the soil (Millero et al., 2006). High saline soils are sometimes recognizable by a white layer of dry salt on the soil surface.

Secondary salinity is the outcome of anthropogenic activities that disrupt the hydrological balance of the soil between water applied (irrigation/rainfall) and water used by crops. Transpiration, and evapotranspiration between irrigation periods can further increase salinity (Doering et al., 1976).

In many irrigated areas, the water table has risen due to excessive amounts of applied water with insufficient drainage. Most of the irrigation system of the world has caused secondary salinity, which not only alters the soil's physicochemical properties but also exerts stress on vegetation which affects yield. However, salinity in the coastal area of Goa has been attributed to salt's upward movement as a seawater intrusion through estuaries and shallow water table resulting in inundation of saltwater in fields during monsoon season and recession during winter and summer causes accumulation of salt on the surface due to upward movement and evapotranspiration.

Types of salinity

Salt-affected soils are grouped into saline soils, sodic soils, and sodic-saline soils. Electrical conductivity (ECe), the ability of a soil solution to carry electrical current, is used to measure soluble salt concentration and is reported in millimhos per centimeter (mmhos/cm) and deciSiemens per meter (dS m^{-1}) (Salinity U.S. Laboratory staff, 1954). At a Soil critical value of ECe >4 dS m^{-1}, crops' yields are restricted and are used to distinguish saline soil. At ECe values between 2 and 4 dS m^{-1}, the growth of only sensitive crops is affected, and at ECe values <2 dS m^{-1}, the effect of salinity is negligible on the crop yield. Some plants grow well in salt-affected coastal areas, shores of backwaters, lakes, and marshy lands. The plant that thrives well in high salt concentrations is called halophytes and grows well with even 200 mM of NaCl (ECe of 20 dS m^{-1}) (Saeed et al., 2014). However, some plants that cannot withstand even at 40 mM (ECe > 4 dS m^{-1}) are called glycophytes (Yadav et al., 2011).

Saline Soils

All soils contain some water-soluble salts, but when these salts occur in amounts that are harmful to the germination of seeds and plant growth, and when soil is characterized by ECe in the root zone exceeding 4 dS m^{-1} at 25°C with exchangeable sodium percentage (ESP) of <15% (40 mM NaCl) and sodium adsorption ratio (SAR) <13 and usually has a pH of less than 8.5 due to higher salt concentration and lesser exchangeable Na^+ ions are called saline soil. Saline soils are in a flocculated state with a permeability equal to or

higher than the average soils and have white soluble salt encrustation on their surface (Bekele, 2021). In India, these soils are commonly known as 'Thur' in Punjab, 'Reh' in Uttar Pradesh, 'Luni' in Rajasthan, and Khazan land in Goa.

Sodic Soils

Sodic soils, also referred to as alkali soils in older literature, are characterized by exchangeable sodium salts capable of inducing alkaline hydrolysis of main bicarbonates. The sodic soils have ECe <4dS m^{-1} at 25^{o}C, ESP >15, and SAR >13. Sodic soil has a pH of more than 8.5 and has very small amounts of free salts in the soil solution. As a result of irrigation, strongly alkaline conditions may follow, with pH values reaching or exceeding 10 observed in these soils. Sodic soils appear brownish-black when deposited with organic matter and are known as black alkali soils. These soils are sticky when wet, nearly impermeable to water, and have a slick look. As they dry, they become hard, cloddy, and crusty. Sodic soils are detrimental to the growth of most plants. They can be reclaimed, but that may be slow and expensive due to the lack of a stable soil structure, which slows water drainage (Gupta & Gupta, 2017). In India, these soils are known as 'Kallar' in Punjab, 'Usar' in Uttar Pradesh, and 'Kshar' in Gujarat.

Saline-sodic Soils

Saline-sodic soils have higher amounts of neutral salts because, in these soils, both soluble salts and exchangeable Na^{+} are high. This soil is characterized by having ECe >4 dS m^{-1} at 25^{o}C, ESP >15, and SAR >13. These soils will have a pH of <8.5 with free salts and exchangeable Na^{+}. The excess salt in the soil keeps the soil flocculated, and upon leaching, as the salt concentration decreases, these soils may act like sodic soil (pH > 8.5) because of the hydrolysis of exchangeable Na^{+}. Both excess salts and sodium levels can adversely affect plant growth in these soils (Osman, 2018).

1.2 Saline soil of Goa

The coexistence of salinity and acidity is usually observed in the coastal soils of Goa. Goa's salt-affected soils are known as 'Khazan' and cover 18000 ha areas with 12000 ha under cultivation during the Kharif season (Korikanthmath et al., 2010) (Fig.1.2). The coastal saline soils in the state are classified into 4 series, viz., Zuari, Colva, Calangute, and Chapora, and these occur prominently in the flood plains of the two major rivers, viz., Zuari and Mandovi, and to a small extent in the flood plain of Chapora, Betula, Talpona,

and Sal River (Swarajyalakshmi et al., 2003). These soils are characterized by low elevation and high water tables. The soils of the Zuari and Mandovi plains (Zuari series) have heavier subsoil horizons and are heavily textured and deep. These soils have high organic matter content and cation exchange capacity (CEC, 25 meq 100 g/1). The pH of the soil is slightly acidic to neutral, and it decreases with depth. The soils of other river plains generally have low CEC and are generally sandy textured. The soils (except Colva, Calangute, and Chapora series) are rich in sesquioxides, highly weathered, and deficient in Ca^{2+}. The soil profile shows concretions of Fe^{3+} and Mn^{2+} and dominant clay minerals in the Zuari series soils characterized by low elevation and high water tables (Swarajyalakshmi et al., 2003). In the Zuari series of Goa, K^{+} status is elevated in soils (Mondal & Singh, 1981; Swarajyalakshmi et al., 2003), and the pH of the coastal saline soil of Zuari was acidic, ranging from 5.0 to 5.3. However, ECe ranges from 10.0 to 15.0 dS m^{-1} (Mondal & Varde, 1979). The soils are rich in Fe^{3+} and Mn^{2+}, sometimes toxic to plants. Cu^{2+} content of the soil is moderate, but B^{3+} and Zn^{2+} are occasionally deficient.

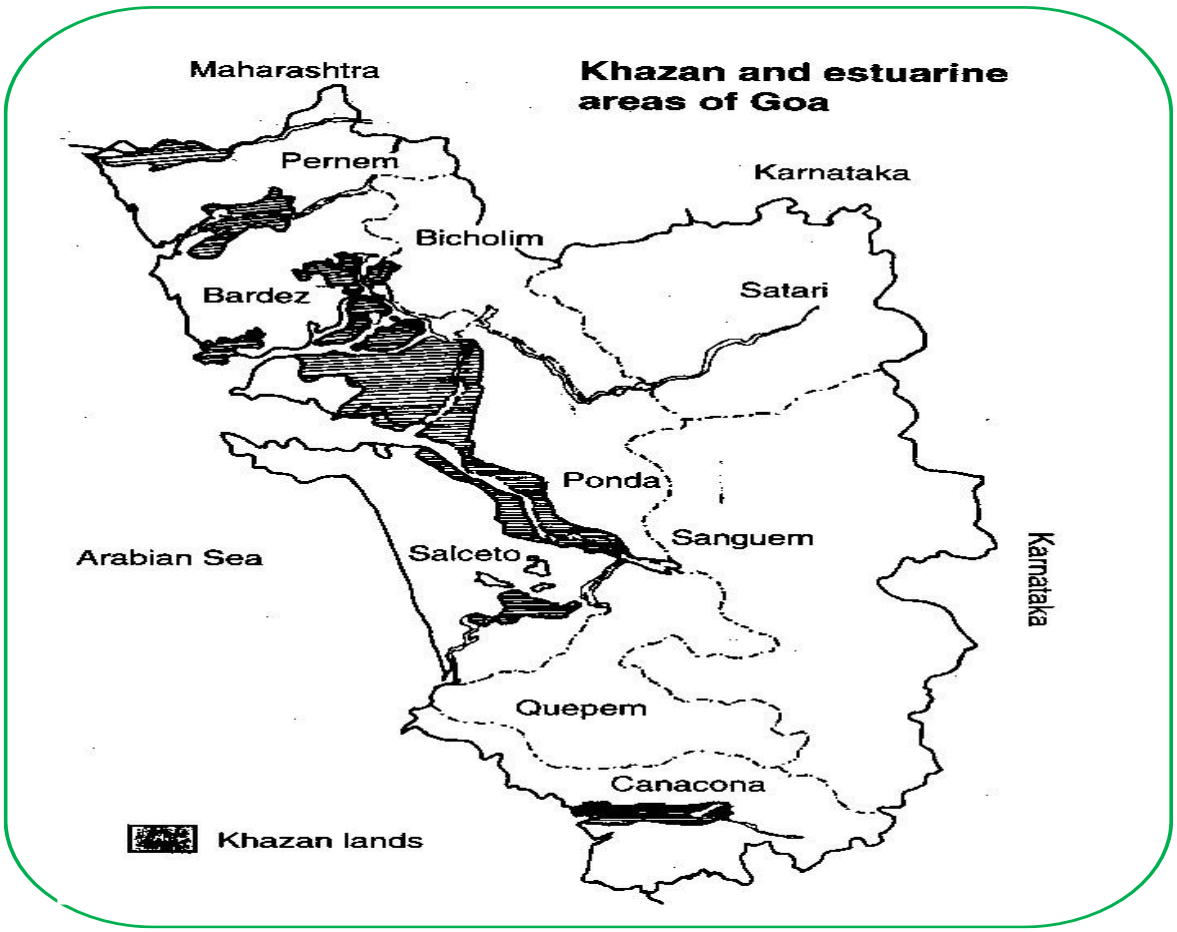


Fig. 1.2: The coastline of Goa (Jagtap, 1994).

1.3 Effect of salt stress on physiological processes in plant

Salinity is a universal abiotic pressure that limits the productiveness of plants and geographical distribution. In many arid and semi-arid regions of the world, despite in-depth physiological studies, minor achievement has been achieved in breeding salinity-tolerant rice varieties.

1.3.1 Effect of salt stress on plant growth

Plant growth could be defined as increasing plant volume or mass and is usually associated with development (cell and tissue specialization). Cell division and expansion are mandatory for growth and development and are severely affected by salinity (Hassan et al., 2021). Salinity restricts water uptake by roots, stunting plants due to reduced cell expansion (Shi et al., 2002; Parida & Das, 2005). Increased levels of salt ions around roots cause loss of cell volume and turgor, which leads to a drop in cell elongation of leaves and stems (Cramer, 2002; Fricke, 2004), decreases in the rate of surface expansion, which can lead to the complete interruption of leaf expansion (Sharma et al., 2019). Limitations in photosynthesis may cause long-term effects on the growth rate in plants under salinity (Munns & Tester, 2008) (Fig. 1.3.1). Salinity changes the activities of enzymes of nucleic acid metabolism (Gomes-Filho et al., 2008), protein metabolism is altered (Dantas et al., 2007), hormonal balance is disturbed (Ashfaque et al., 2014), and the utilization of seed reserves is reduced (Othman et al., 2006) which reduced the plant productivity (Takemura et al., 2000). Excess salt reduces the fresh and dry weight of leaves, stems, and roots (Takemura et al., 2000), germination rate, plumule growth, seedling length, length of the radicle, and seed vigor in *Z. mays* exposed to 240 mM NaCl (Khodarahmpour et al., 2012; Parihar et al., 2015).

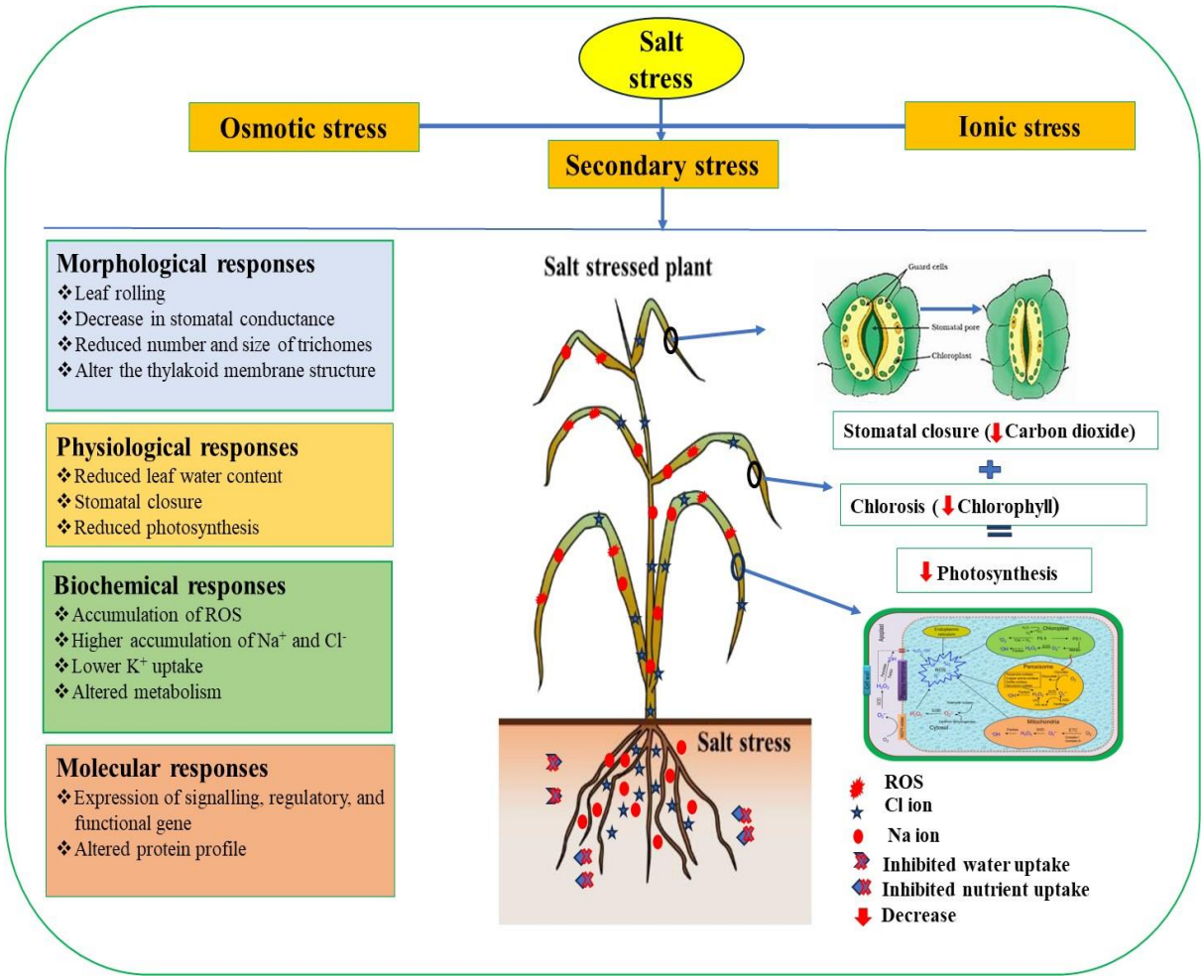


Fig. 1.3.1: Morphological, physiological, biochemical, and molecular responses under salinity stress. Accumulation of sodium (Na^+) and chloride (Cl^-) ions in the soil inhibit water and nutrient absorption, leading to impaired cellular water status (osmotic stress) and an extreme case of ion toxicity (Chele & Tugizimana et al., 2021).

Phytohormones such as abscisic acid (ABA) and gibberellic acid (GA) play a significant role in the regulation of shoot and root growth under salt stress (Achard et al., 2006; Munns & Tester, 2008). A study by Achard et al. (2006) showed that salt induces ABA and ethylene signaling pathways regulates the growth of plants through activating functions of DELLA proteins. A study by Yang et al. (2019) showed that the endodermis is the point of crosstalk between ABA and GA pathways (GA antagonizes the ABA pathway), which causes the regulation of root growth under salt stress.

1.3.2 Effect of salt stress on external and internal leaf morphology

The epidermis is the outermost tissue of all plant organs and acts as a first contact point with their surroundings. It plays a vital role in all plant-environment interactions and is essential for maintaining physiologically favorable conditions (Bailes & Glover, 2018). In the aerial organs of most terrestrial plants, the epidermis is patterned with trichomes, which are epidermal outgrowths with diverse roles in the defense against biotic and abiotic stresses. The epidermis also contains stomata, which are epidermal pores that regulate gas exchange and contribute directly to controlling water status. The cuticle covering the epidermis's surface is a hydrophobic layer consisting of cutin and waxes that prevent uncontrolled water loss (Riederer & Schreiber, 1995). As a result of their function in limiting water losses, specialized structures in the epidermis are promising targets to improve the stress tolerance and Water Use Efficiency (WUE) of major crops (Rasool et al., 2013; Bailes & Glover, 2018). In addition to abiotic stress tolerence, trichomes also play a role in tolerance to biotic stress (Tian et al., 2017; Bekele, 2021). Several studies have indicated that trichomes are major sites for the biosynthesis of secondary metabolites and stress proteins (Harada et al., 2010). Trichomes also help plants adapt to stress by developing glutathione and sulfur-dependent defense mechanisms and redox regulation (Wienkoop et al., 2004). Stomata are the main structures responsible for gas exchange control, and salt stress affects not only stomatal opening but also their size and density, resulting in a decrease in stomatal conductance. Consequently, transpiration rates (water loss) and photosynthesis (CO_2 uptake) are also reduced. A reduction in stomatal density may partially compensate for the trade-off between plant growth and adaptation (Ouyang et al., 2010; Orsini et al., 2011) and therefore be advantageous under saline stress (Silva et al., 2009; Li et al., 2017).

Chloroplast morphology is related to productivity (Burundukova et al., 2003). Rice mesophyll tissues are among the most challenging structures to evaluate by conventional microscopy because their mesophyll cells are smaller (Chonan, 1978) and have a higher chloroplast density (Sage & Sage, 2009; Oi et al., 2017) compared to other crops. The unique shape of rice mesophyll cells, which is discoid with several lobes covered by chloroplasts inside of the cell periphery, is thought to enhance CO_2 diffusion from the intercellular space to the stroma (Oi et al., 2017). Therefore, accurately assessing mesophyll morphology is essential for estimating photosynthetic ability (Burundukova et al., 2003). Under salinity stress, the chloroplast structure in the leaf mesophyll cell is altered, affecting photosynthetic activity (Yamane et al., 2008). These structural changes

are particularly prominent in the thylakoids (Omoto et al., 2016), which become swollen under salinity stress (Rahman et al., 2000; Yamane et al., 2004; Yamane et al., 2008). A substantial reduction in photosynthesis has been associated with decreased total chlorophyll content and distortion in chlorophyll ultrastructure (Zhang & Shi et al., 2013).

1.3.3 Effect of salt stress on Water Potential

The most used parameter to characterize plant water status is water potential (Ψ). Total water potential (Ψ) has four components: the Osmotic potential (ψs), Pressure potential (ψp), Matric potential (ψm), and Gravitational potential (ψg)

$$\Psi_{total}=\psi s+\psi p+\psi m+\psi g$$

Osmotic potential (ψs) results from dissolved solutes in cell sap and is proportional to solute concentration and inversely proportional to cell water volume. ψs in plants are always negative and decrease as solutes concentrate during dehydration. Pressure potential is a measure of tissue turgor produced by the diffusion of water into the protoplast of cells enclosed by largely inelastic cell walls. Matric potential (ψm) arises from the action, on water, of electrostatic forces of attraction associated with the cell wall and colloidal surfaces and of the capillary forces related to narrow transport vessels. In plants, it is considered to be negligible. Gravitational potential (ψg) results from gravitational forces acting on the water within plants. In most situations, total plant water potential is considered to be the sum of the Pressure Potential (ψp) and Osmotic potential (ψs). Leaf water potential (ψL) controls stomatal conductance, which affects transpiration and photosynthesis, and affects root water uptake driven by the potential difference between leaf and soil water (Cramer, 2002; Vos & Haverkort, 2007).

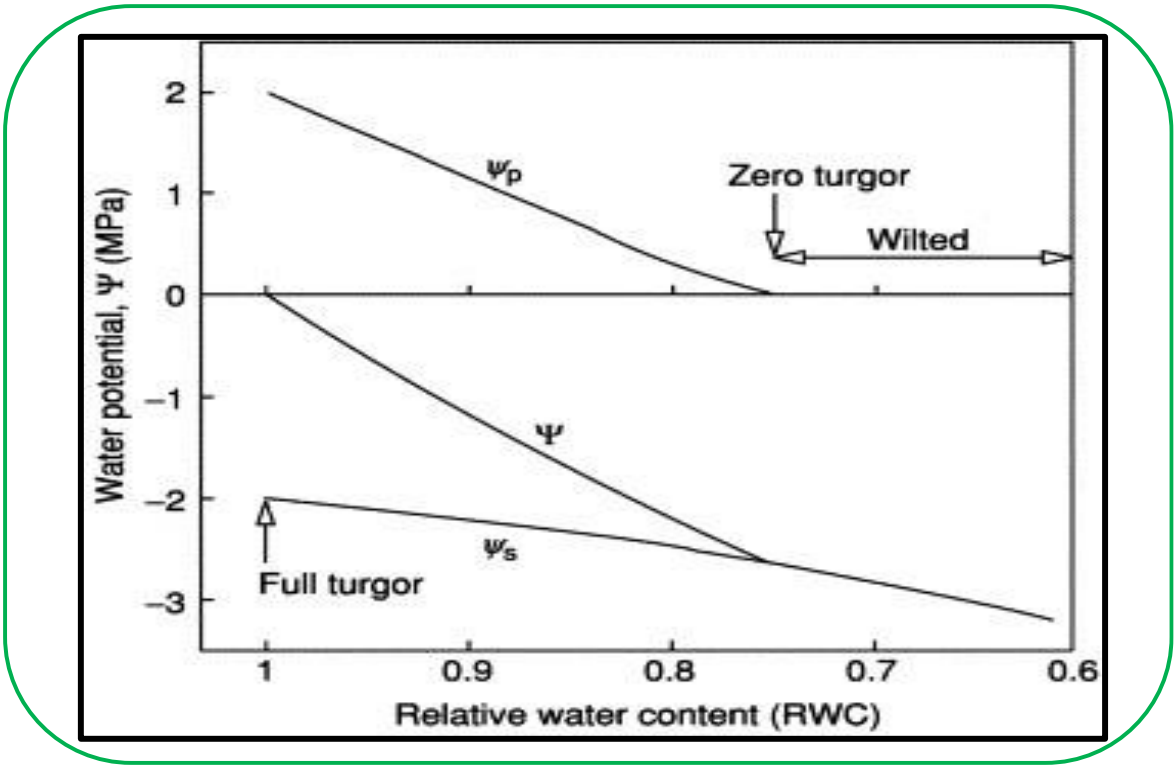


Fig. 1.3.3.1: Diagram showing relationships between Total water potential (Ψ), Pressure potential (ψp), Osmotic potential (ψs), and Relative water content (RWC) as a cell or tissue loses water from a fully turgid state (Vos & Haverkort, 2007).

In saline soil, plants cannot take up enough water to meet their evaporative demands because of the low osmotic potential of the soil water. Thus, higher transpiration induces water loss from the plant leaves and reduces ψL. The reduction in ψL induces stomatal closure, which prevents water vapor and CO_2 transport, and thus, photosynthesis and transpiration decrease. Based on these physiological processes, it has been suggested that ψL can be used to develop appropriate irrigation management to improve crop production in saline areas (Fricke et al., 2006). Salinity altered plant water relations mainly by an osmotic adjustment (Çulha & Çakirlar, 2011). Maintenance of plant water status is a fundamental phenomenon for the maintenance of normal growth of plants under stressful environments (Ali & Ashraf, 2011). It has been argued that salt-tolerant plants decreased the hydraulic conductance of their roots, thereby reducing the delivery of (salty) water to the shoot (Vysotskaya et al., 2010) and resulting in reduced water potential in their leaves (Gama et al., 2009).

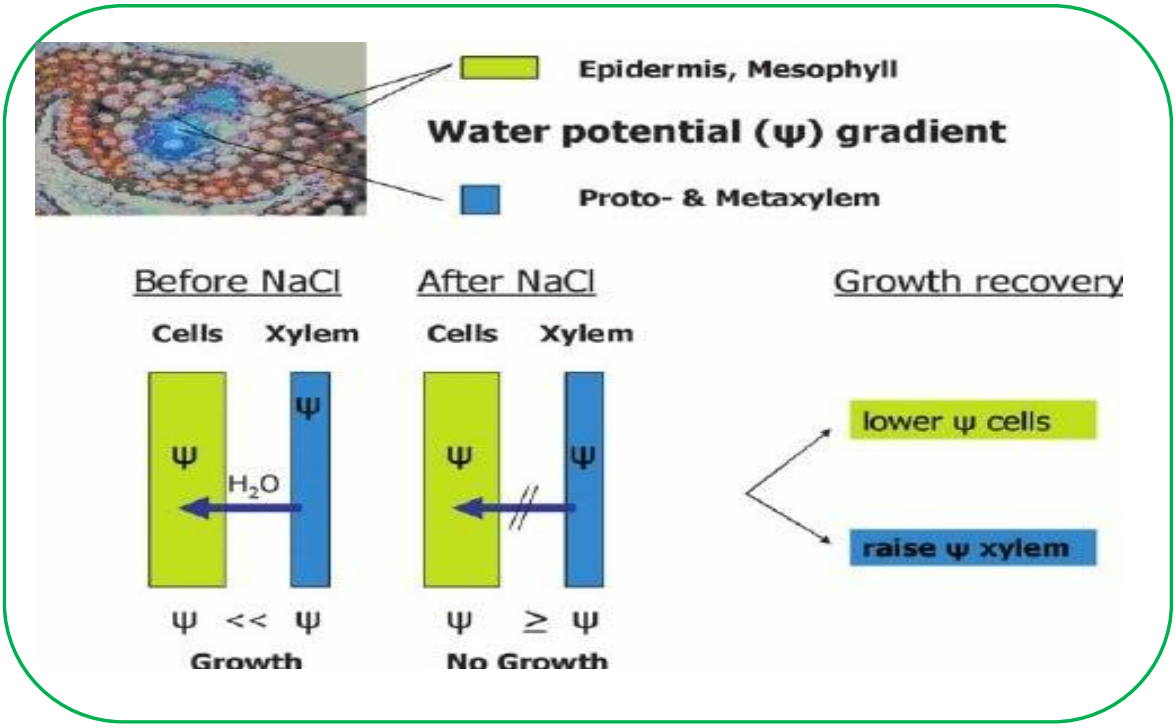


Fig. 1.3.3.2: Diagram showing relationships between Total water potential (Ψ), before and after NaCl treatment and recovery in epidermis mesophyll cell and protoxylem and meta xylem (Fricke et al., 2006).

1.3.4 Effect of salinity on photosynthesis

Photosynthesis is the process of converting light energy to chemical energy and storing the energy in the bonds of sugars and other organic compounds.

$$6CO_2 + 6H_2O \xrightarrow[\text{Chlorophyll}]{\text{Sunlight}} C_6H_{12}O_6 + 6CO_2$$

Photosynthesis is one of the major metabolic processes which are sensitive to environmental stresses. Photosynthesis consists of two major processes: the light-dependent electron transport chain and the light-independent carbon fixation cycle.

Light-dependent electron transport chain

Light reaction of photosynthesis occurs in the thylakoid membranes of the chloroplast, which are large organelles bounded by a double membrane (Fig. 1.3.4.1). In addition to the inner and outer membranes of the envelope, chloroplasts have a third internal membrane system called the thylakoid membrane. The thylakoid membrane forms a linkage of compressed discs called thylakoids, normally arranged in grana stacks. Three membranes divide chloroplasts into three distinct internal compartments: the

intermembrane space, the stroma, and the thylakoid lumen. Thylakoid membranes have pigments, for example, chlorophylls and carotenoids, which absorb light, whereas carotenoid such as (β-carotene, zeaxanthin, and tocopherols) also play an essential photo-protective role in all photosynthetic organisms scavenging Reactive Oxygen Species (ROS) or by dissipating excessive energy in the form of heat (Mittler, 2002). High carotenoid content favors better saline adaptation of sugarcane plants (Gomathi & Rakkiyapan, 2011).

Highly structured multi-subunit protein-chlorophyll complexes of Photosystem I (PSI) and Photosystem II (PSII), arranged in supra-molecular assemblies embedded in thylakoid membranes contains pigments that absorb light to oxidize water to molecular oxygen (Fig. 1.3.4.1B). Thylakoid membrane stacking, is an essential factor in the regulation of photosynthesis, and depends on Vander Waals forces, lateral segregation of protein complexes within the membrane, cations mediated electrostatic interaction between membrane and steric hindrance (Dekker & Boekema, 2005). The shape of grana is also crucial in thylakoid stacking and depends on the percentage and type of lipids as well as the connection between the protein complexes and the lipids (Dekker & Boekema, 2005).

Salinity stress induces swelling of thylakoids and damaged cell membranes (Yamane et al., 2008). Salt-grown plants affect the chloroplast ultrastructure, with a pronounced swelling of thylakoids, likely due to the unbalance of the osmotic equilibrium of organelles (Goussi et al., 2018). Modifications in the lamellar organization, resulting in chloroplast shrinkage (Papadakis et al., 2007), swelling of chloroplast lamellae, and an unrecognizable grana structure which reduced chlorophyll content under salt stress are also reported (Rasool et al., 2013). Arabidopsis seedlings grown in the presence of salt exhibit swollen chloroplasts with less developed granum structures (Peharec et al., 2013). Salt stress-induced destruction of the chloroplast envelope and increased plastoglobuli in thylakoid membranes have been reported in cucumber leaves (Shu et al., 2012).

Light energy is captured by a series of pigments localized in LHC and Reaction Centers (RC); these reactions take place by involving the coordination between numerous proteins that are organized in four major multi-subunit protein complexes: the PSI, PSII, cytochrome b6/f (cyt b6/f) complex, and the ATP synthase (Hippler et al., 2001) (Fig. 1.3.4.1 C). Photosystems I and II absorb light at wavelengths of 700 nm (far-red) and 680 nm (red), respectively, and are spatially distributed in the ratio 1: 1.5 in the thylakoid membrane (Fig. 1.3.4.1C). PSII centers are localized in appressed membrane regions,

called the grana stack of the thylakoid membranes. In contrast, PSI centers are mainly localized in the non-appressed regions or stroma lamellae (Koochak et al., 2019). Proteins that form the PSII core complex in plants include D1 and D2 (i.e., PsbA and PsbD), two small subunits forming Cyt b559 (9 and 5 kDa), core antenna proteins CP43 and CP47 (also known as PsbC and PsbB, respectively) (Komenda et al., 2012).

LHC II consist of six polypeptides called Lhcbl (CP20), Lhcb2 (CP27.5), Lhcb3(CP27.5), Lhcb4, Lhcb5 and Lhcb6. Lhcbl-3 LHCII apoprotein binds eight Chl 'a', six Chl 'b' and four carotenoids and exist as heterotrimers. The other chlorophyll a/b-binding proteins, Lhcb4 (CP29), Lhcb5 (CP26), and Lhcb6 **(**CP24) exist as monomers (Janson, 1994; Pan et al., 2011). Photosystem II is the definitive source of electrons for photosynthesis, and its function is also too prone to damage by various stress factors. It has been reported that salinity causes a reduction in antenna size, as well as cytochrome Cyt b6f (Parida & Das, 2005). Differences have also been shown in the polypeptide composition of PSII in salt-tolerant and salt-sensitive plant species (Wang et al., 2007) and salinity-induced proteolysis of thylakoid membrane proteins and reduction in SDS-PAGE polypeptide contents (Misra et al., 1997).

PSI catalyzes the final stage of NADPH formation from $NADP^+$ in the light reaction. The enzymatic heart of the PS I complex has 3 subunits known as PsaA, PsaB liganded with the prosthetic groups, P700, A_0, A_1, and Fx and Fa and Fb iron-sulfur clusters are found in PsaC. PSI core complex binds to the membrane-bound peripheral antenna, called LHCI, and this antenna consists of four polypeptides called Lhca1 (22kDa) and Lhca2 (23kDa), Lhca3 (25kDa), and Lhca4 (22kDa) (Jansson, 1994). Chl 'a' molecule lies at the center of the structure, which absorbs light maximally at 700 nm (P700). Upon excitation-either by direct absorption of a photon or exciton transfer-P700* transfers an electron through the complex, catalyzing the reduction of $NADP^+$ via ferredoxin: $NADP^+$ reductase.

The cytochrome b_6f complex is consistently distributed among the stroma and the granum lamellae. The cytochrome b_6f complex consists of four different integral polypeptides, cyt b6 (cyt b563), cyt f, iron-sulfur protein (a 2Fe-2S protein), and component IV consists of a 24kDa cytochrome b6 subunit, 17kDa subunit IV, 19kDa Rieske iron-sulfur protein, and 31kDa c-type cytochrome-f subunit. The function of this complex is to permit electrons from PSII to PSI by oxidizing PQH_2 and reducing plastocyanin, also by transporting H^+ from the stroma into the thylakoid lumen (Fig. 1.3.4.1 C). The electrons are then shifted from PSI down the electron transport chain to

diminish $2NADP^+$ to 2NADPH. Throughout this process, a proton gradient is produced across the thylakoid membrane that drives the synthesis of ATP.

Salinity is reported to inactivate both PS II and PSI-mediated electron transport and damage the oxygen-evolving machinery of PS II (Allakhverdiev et al., 2000). Salt stress reduces the utilization of trapped photons in transferring electrons from Q_A to Q_B and beyond Q_B to the electron transport chain. Thus, it causes over-reduction of the Plastoquinone pool (PQH_2) under salinity stress (Rastogi et al., 2020). Salt stress decreases in the Performance Index on an Absorption Basis (PIABS), which is linked with a reduction in active reaction centers, trapping, and transport of electrons to ETC (Küpper et al., 2019). Salinity stress mediates a decrease in Fo and Fm due to a reduction in the electron transport from P680 to Q_A (Gulzar et al., 2020). Changes in Fo and Fm are known due to salinity (Yamane et al., 2004). Reduced Fv/Fm and the PSII efficiency (ΦPSII) are more significant in salt-sensitive than salt-tolerant (Kalaji et al., 2012). In saline conditions, a decrease in photochemical quenching (qP) and the electron transport rate (ETR), while an increase in non-photochemical quenching parameters (qNP) has been reported in maize seedling (Qu & Hong, 2012). The higher qNP value due to salinity stress has been investigated in halophytes plant such as *Arthrocnemum macrostachyum* (Redondo-Gómez et al., 2010; Trotta et al., 2012), *Sarcocornia fruticose* (Redondo-Gómez et al., 2010), and in *Atriplex centralasiatica* (Qiu et al., 2003), showing higher non-photochemical quenching that dissipates excess excitation energy of PSII in the form of heat as a first line of defense thus preventing the formation of potentially cytotoxic reactive ROS.

ATP and NADPH generated from the light reaction in the thylakoid membrane flow to the stroma to drive the enzyme-catalyzed reduction of atmospheric CO_2 to carbohydrates (Fig. 1.3.4.2B). The two major products of the photosynthetic fixation of CO_2 are starch and sucrose. The former is a reserve polysaccharide that accumulates transiently in the chloroplast, and the latter is a disaccharide exported from leaves to the developing and storage organs of the plant. The Calvin-Benson cycle (Bassham & Calvin, 1950) completes in 3 stages. First is the carboxylation step, during which CO_2 is accepted by Ribulose-1,5-Bisphosphate (RuBP) to produce two molecules of a three-carbon compound, 3-Phosphoglyceric acid (3-PGA) and is catalyzed by RuBP Carboxylase/Oxygenase (RuBisCO). The second stage is the reduction of 3-PGA is converted to Glycerldehyde 3- phosphate (G3P) by utilizing the ATP and NADPH from the thylakoid reaction and the third stage is regeneration, during which the CO_2 acceptor

ribulose-1, 5-bisphosphate re-forms from is converted into G3P by using ATP and NADPH (Fig. 1.3.4.2B).

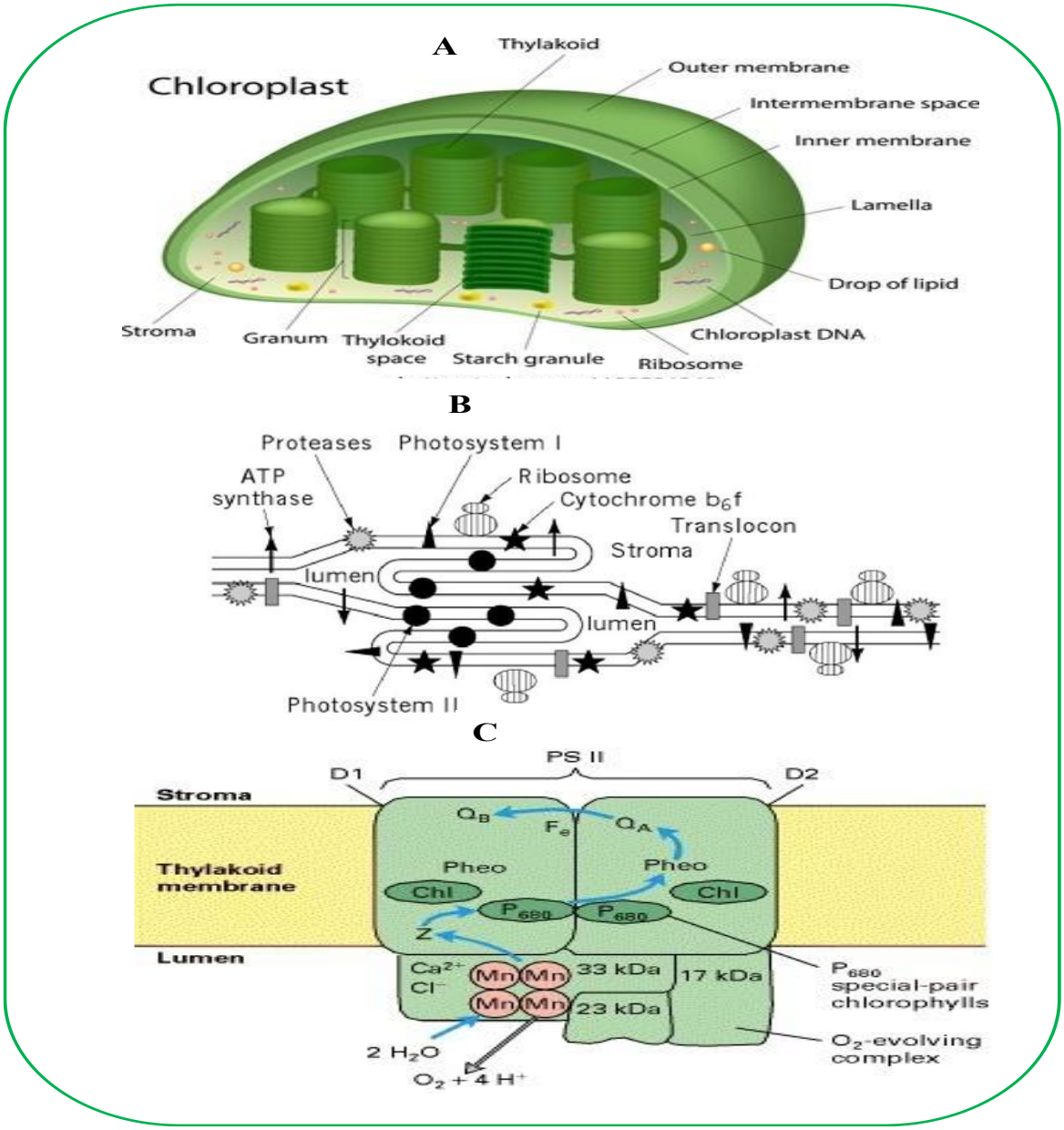


Fig. 1.3.4.1: Schematic diagram of chloroplast representing the organization of the membranes in the chloroplast (A), the protein complexes of the thylakoid membranes (B), and photosystem II complex (C) (yaclass. in; what-when-how.com; and Lodish et al., 2008, respectively).

Photosynthesis is the primary method liable for biomass synthesis and primary production. The decrease in photosynthesis induced by salt stress could be due to the stomatal and nonstomatal factors that are accompanied by decreased transpiration and

CO_2 assimilation in plant species in a wide range of salinity (Dingkuhn et al., 1992; Parida et al., 2004; Munns and Tester, 2008). Excess salt in plants harms the cell membrane and organelles of the plant, resulting in a reduction in plant physiological mechanisms such as the net photosynthesis rate (*Pn*), stomatal conductance (*gs*), transpiration rate (*E*), and intracellular carbon dioxide (*Ci*) which lead to plant cell death in rice (Hussain et al., 2018; Akram et al., 2019). The reduction in stomatal conductance results in the limited availability of CO_2 for carboxylation reactions and decreased photosynthesis rate (Chaves et al., 2009). Furthermore, salt stress in plants increases the osmotic potential and reduces water availability leading to cell membrane dehydration and decreased CO_2 permeability (Riaz et al., 2019). In addition to gas exchange characteristics, and stomatal properties salinity is also reported to minimize photosynthetic pigments (Zheng et al., 2013; Hassan et al., 2021).

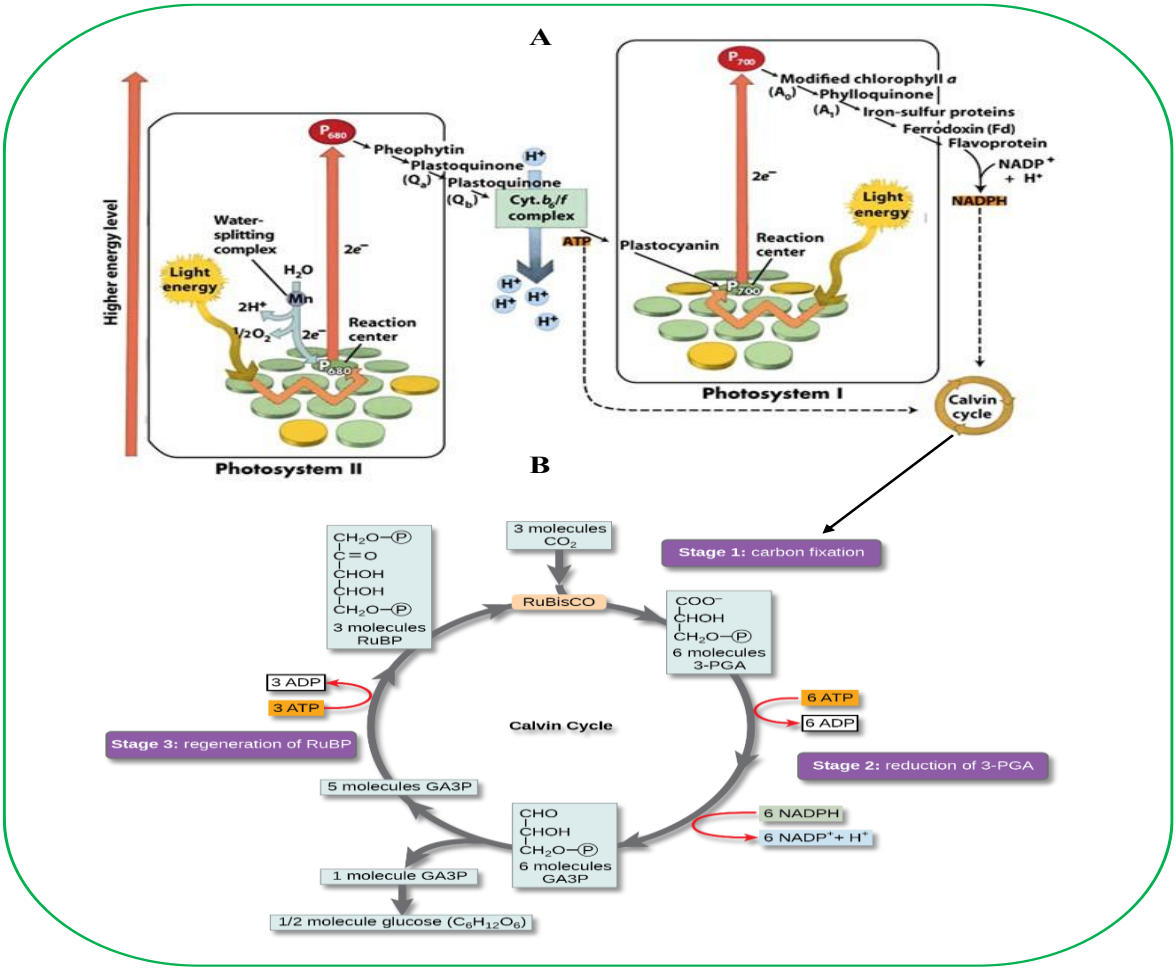


Fig. 1.3.4.2: A schematic diagram of non-cyclic photophosphorylation (Z scheme) (A) and carbon dioxide fixation (B) of photosynthesis in plants (Biology LibreTexts; neetlessons.com respectively).

1.4 Biochemical response

1.4.1 ROS production under salinity

ROS are a group of free radicals, reactive molecules, and ions that are derived from O_2. It has been estimated that about 1% of O_2 consumed by plants is diverted to produce ROS in various subcellular loci such as chloroplasts, mitochondria, and peroxisomes (Asada & Takahashi, 1987) (Fig. 1.4.1). ROS are well documented for playing a dual role as both deleterious and beneficial species depending on their concentration in plants. Salt stress prevents carbon metabolism due to stomatal closure, which lowers CO_2 availability for the Calvin cycle, leading to the depletion of the pool of oxidized $NADP^+$, and electrons are transferred to O_2 to generate $O_2^{\bullet-}$ (Mehler, 1951). ROS are produced through metabolic reactions where different enzymatic and non-enzymatic pathways are involved. Stepwise monovalent reduction of O_2 leads to $O_2^{\bullet-}$, H_2O_2, and OH•, whereas energy transfer to O_2 leads to the formation of $^1O_2$. $O_2^{\bullet-}$ is easily dismutation to H_2O_2 either non-enzymatically or by superoxide dismutase (SOD) catalyzed reaction to H_2O_2. H_2O_2 is converted to H_2O by catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX) (Fig. 1.4.1) (Hasanuzzaman et al., 2020).

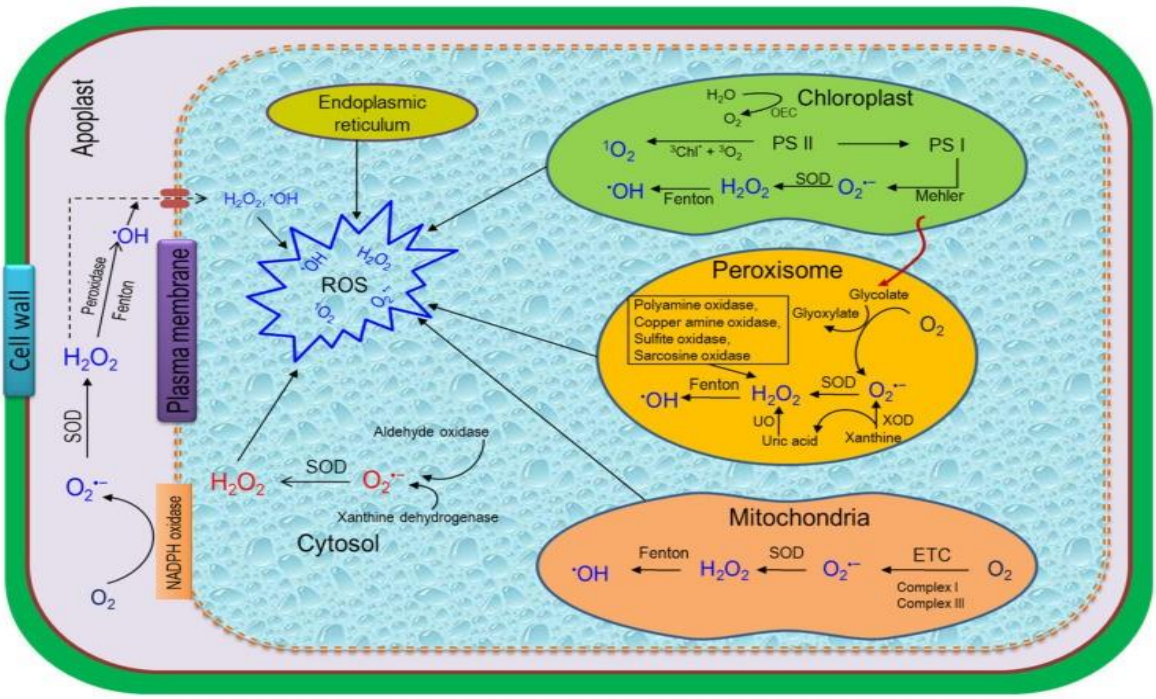


Fig. 1.4.1: Schematic representation of the generation of ROS process and localization in various organelles in the plant cell (Hasanuzzaman et al., 2020).

TYPES OF ROS

Singlet Oxygen ($^1O_2$): Singlet oxygen $^1O_2$ is the first excited electronic state of O_2 and is an abnormal ROS because it is not related to electron transfer to O_2. Insufficient energy dissipation during photosynthesis can lead to chlorophyll (Chl) triplet state formation. The Chl triplet state can react with $3O_2$ to form very reactive $^1O_2$.

$$\textbf{Chl Light} \rightarrow \textbf{3Chl… (1)}$$

$$\textbf{3Chl + 3O}_2 \rightarrow \textbf{Chl+ }^1\textbf{O}_2\textbf{… (2)}$$

It has been found that the formation of $^1O_2$ during photosynthesis has a powerful, damaging effect on PSI and PSII as well as on the whole photosynthetic machinery. $^1O_2$ is an oxidizing agent for a wide range of biological molecules and can react with proteins, pigments, nucleic acids, and lipids, and thought to be the most important species responsible for light-induced loss of PSII activity which may trigger cells death (Asada & Takahashi, 1987; Kim et al., 2021).

Superoxide Radical ($O_2^{\bullet-}$): Superoxide radical is constantly generated in the chloroplasts due to partial reduction of O_2 during electron transport under conditions of over-reduction. The superoxide radical ($O_2^{\bullet-}$) forms electrons in different cell compartments, including chloroplasts, peroxisomes, apoplast, the mitochondrial transport chain, and the plasma membrane (Janků et al., 2019). O_2 reacts with the different ETC components to give rise to the $O_2^{\bullet-}$. It is usually the first ROS to be formed during the Mehler reaction.

Hydrogen Peroxide (H_2O_2): Hydrogen peroxide, a moderately reactive ROS, is formed when $O_2^{\bullet-}$ undergoes univalent reduction. Hydrogen peroxide is produced in plants via two possible pathways: dismutation of $O_2^{\bullet-}$ with the involvement of SOD (Queval & Noctor, 2007) and via oxides such as amines and oxalate oxidases (Sharma et al., 2012). In chloroplasts, hydrogen peroxide is produced by the dismutation of superoxide radicals in a reaction catalyzed mainly by superoxide dismutase (Asada et al., 1974). In mitrochondrial H_2O_2 is generated when cytochrome ***c*** oxidase interacts with O_2. Sometimes, it can occur both non-enzymatically and enzymatically by being dismuted to H_2O_2 under low pH conditions.

$$\textbf{2O}_2^{\bullet-} \textbf{ + 2H}^+ \xrightarrow{\textbf{SOD}} \textbf{H}_2\textbf{O}_2 \textbf{ + O}_2$$

Hydroxyl radical ($OH^{\bullet}$) and Hydroxyl anions (OH^{-}): The hydroxyl radical, $OH^{\bullet}$ is the neutral form of the hydroxide ion (OH^{-}). Hydroxyl radicals are highly reactive and consequently short-lived in normal conditions. Hydroxyl radical ($OH^{\bullet}$) is the most reactive and toxic ROS known. It is generated at neutral pH by the Fenton reaction in between H_2O_2 and $O_2^{\bullet-}$ catalyzed by transition metals like Fe (Fe^{2+}, Fe^{3+}).

$$\mathbf{O_2^{\bullet-} + Fe^{3+} \rightarrow {}^1O_2 + Fe^{2+}}$$

$$\mathbf{Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH- + OH\bullet \ (Fenton\ Reaction)}$$

Lipoxygenase (LOX): LOX is nonheme iron-containing dioxygenase widely distributed in plants and animals. LOX catalyzes the oxidation of polyunsaturated fatty acids containing a ***(***Z**,** Z***)***-1,4-pentadiene structure (linoleic, linolenic, and arachidonic acid). During salt stress, the cell wall-localized LOX causes hydroperoxidation of polyunsaturated fatty acids (PUFA), making it the active source of ROS like $OH^{\bullet}$, $O^{\bullet-}{}_2$, H_2O_2, and $^1O_2$ (Bhattacharjee, 2019). In plants, linolenic and linoleic acids are the most common substrates for LOX (Siedow, 1991).

Salinity stress-induced physiological drought results in low availability of CO_2, resulting in decreased carbon dioxide fixation and $NADP^+$ formation by Calvin's cycle leading photoreduction of O_2 at PSI to produce $O_2^{\bullet-}$, detoxified to H_2O_2 and O_2 by superoxide dismutase via the Mehler reaction (Chen et al., 2004; Ozgur et al., 2013; Gulzar et al., 2020). Salinity induced higher H_2O_2 content in *Arabidopsis* compared to *T. salsuginea* (halophyte plants) reported by Wiciarz et al. (2015). Salt-tolerant tomato cultivars have less generation of H_2O_2 compared to sensitive cultivars under salt stress (Hameed et al., 2021). Salt tolerance *Medicago truncatula* genotypes showed lower content of H_2O_2 under salt stress conditions (Mhadhbi et al., 2013). Hernández et al. (2000) showed higher apoplastic H_2O_2 levels in a salt-sensitive cultivar of pea than in a salt-tolerant cultivar. Salt-stress-induced increased production of O_2^- and H_2O_2 in rice (Jiang et al., 2020; Simon & Yusuf, 2020). Furthermore, higher H_2O_2 content in salt-sensitive sunflower variety as compared to salt tolerance under salt stress (Lalarukh & Shahbaz, 2020). However, halophyte species have efficient mechanisms to control the production of ROS or detoxify them compared to glycophytes, either through the dissipation of excess excitation energy to alternative electron sinks, such as the plastid terminal oxidase (Uzilday et al., 2015).

1.5 Oxidative damage caused by ROS

Oxidation is a chemical reaction that creates free radicals and causes cell death, leading to a chain reaction. However, the balance between ROS production and scavenging is disturbed under several stressful conditions, such as salinity, drought, high light, metal toxicity, and pathogens. Increased levels of ROS can cause damage to biomolecules such as lipids, proteins, and DNA (Fig. 1.5). These reactions can alter intrinsic membrane properties like fluidity, ion transport, loss of enzyme activity, protein cross-linking, protein synthesis inhibition, DNA damage, and ultimately cell death.

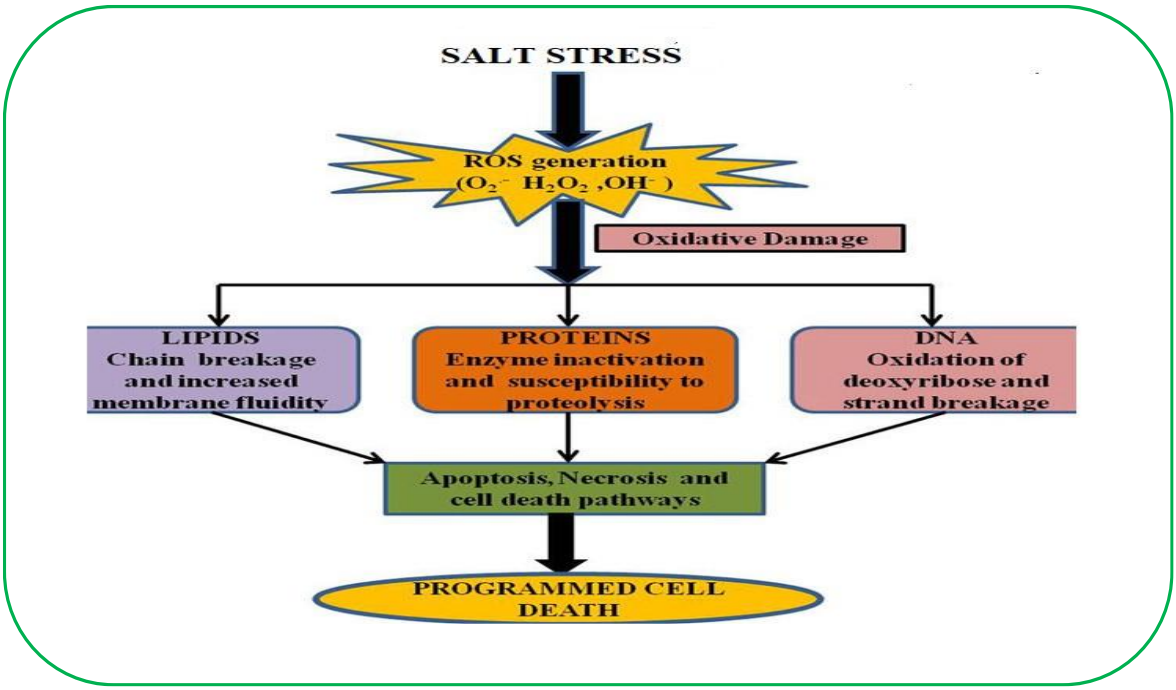


Fig. 1.5: Oxidative damage to lipids, proteins, and nucleic acid by ROS induced by salt stress (Awasthi et al., 2015).

1.5.1 Lipid peroxidation:

Lipid peroxidation (LPO) can be described generally as a process under which oxidants such as free radicals attack lipids containing carbon-carbon double bond(s), especially Polyunsaturated Fatty Acids (PUFAs) (Fig.1.5.1). Increased lipid peroxidation under stresses has been reported in parallel with high ROS production. LPO form polyunsaturated precursors that include small fragments of hydrocarbons, such as ketones, MDA, etc., and compounds related to them. Some of these compounds react to form colored products called Thiobarbituric Acid Reactive Substances (TBARS) with Thiobarbituric Acid (TBA) (Sergio et al., 2012). Malondialdehyde (MDA) is one of the

final products of the peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage. MDA is increased as the salinity level increases (Sergio et al., 2012). Fatty acids are an important component of membrane lipids and are considered essential for salt tolerance in plants (Azachi et al., 2002). The unsaturated (double) bond between two carbon atoms and the ester linkage between glycerol and fatty acid are two typical ROS attacks on phospholipid molecules. The PUFAs present in the membrane phospholipids are susceptible to ROS assault. It has been suggested that one of the primary mechanisms for adaptation to salt stress is an increase in the degree of unsaturation of fatty acids of membrane lipids (Fujii et al., 2001; Guo et al., 2019). Zhao and Qin (2005) reported that the application of exogenous fatty acids could attenuate salt-induced injury in the roots of barley seedlings. Many reports have suggested that lipids might be involved in the protection against salt stress and temperature stress (Menard et al., 2017). The enhanced balance of highly polyunsaturated fatty acids, primarily linolenic and eicosapentaenoic acid, was considered the individual of the relative salt tolerance (Hajlaoui et al., 2009).

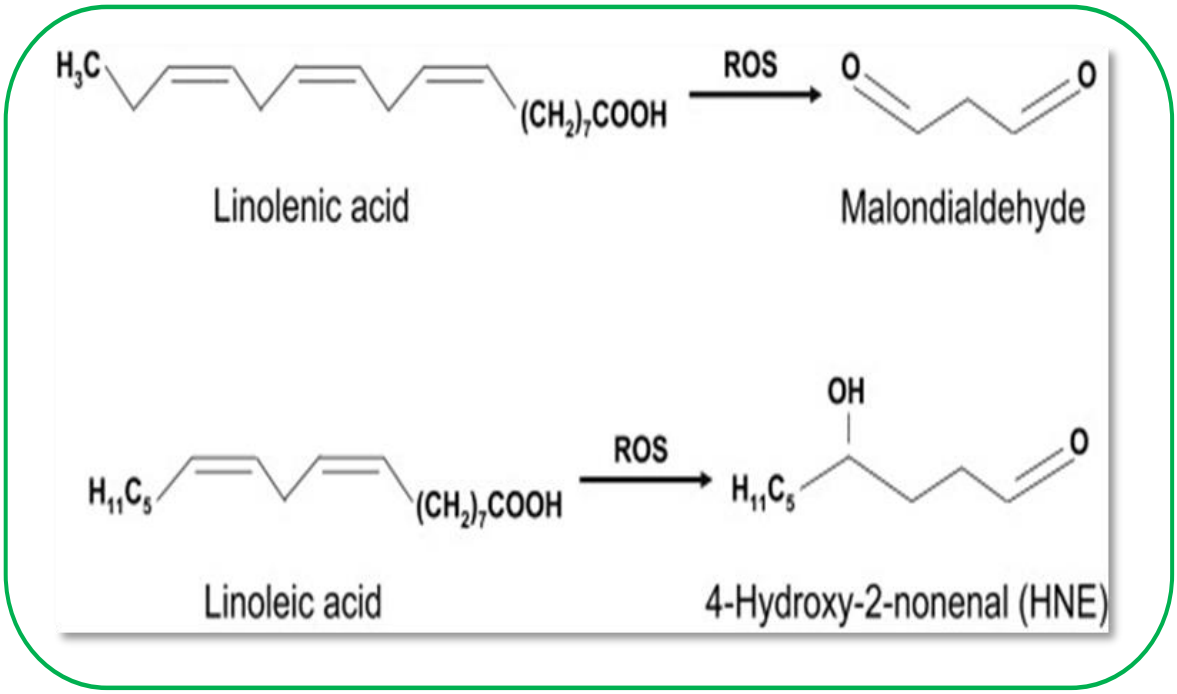


Fig. 1.5.1: Lipid peroxidation in plants caused by ROS (Gills & Tuteja, 2010).

Reports suggest peroxidation of lipids by salinity in sensitive genotypes compared to tolerant genotypes (Liang et al., 2003; De Azevedo Neto et al., 2006; Ashraf & Ali, 2008; Sayyad et al., 2016). Similarly, MDA was reported due to salt stress in sweet peppers

(Abdelaal et al., 2020) in mung bean following NaCl exposure (Ahmad et al., 2019). Increased MDA content was also noted in maize plants under salt stress (Arora et al., 2020).

1.5.2 Protein oxidation:

Proteins are the key players in most cellular events; hence, functional conformations of cellular proteins are essential to survive under stress conditions (Timperio et al., 2008; Ghosh & Xu, 2014). The attack of ROS on proteins may cause modification of proteins in a variety of ways, and some are direct and others indirect (Fig. 1.5.1). Direct modification involves modulation of a protein activity through nitrosylation, carbonylation, disulfide bond formation, and glutathionylation. Proteins can be modified indirectly by conjugation with fatty acid peroxidation breakdown products (Yeilaghi et al., 2012). Tissues injured by oxidative stress generally contain increased concentrations of carbonyl content, which are widely used markers of protein oxidation (Ahmad et al., 2019).

Thiol groups and sulfur-containing amino acids are very susceptible sites for attack by ROS. Oxidative modifications in proteins (such as oxidation of thiol residues and formation of carbonyl derivatives) caused by oxidative stress due to environmental factors have a special significance in eco-toxicological assessments (Braconi et al., 2011). The protein-carbonyl content has been a general indicator and the most used marker of ROS-mediated protein oxidation in abiotic-stressed plants (Wei et al., 2009).

In plants, cellular compartments such as cytosol, chloroplasts, peroxisomes, nucleus, and mitochondria may exhibit carbonylated proteins (Rajjou et al., 2008). The highest concentration of oxidatively modified proteins has been reported in *T. aestivum* leaves (Bartoli et al., 2004) and legume nodules (Matamoros et al., 2013). A prominent increase in the protein carbonyl concentration of salt-sensitive than salt-tolerant genotype in rice under salt-stressed seedlings has been reported (Sharma & Dubey, 2019). Keshavkant et al. (2012) have also reported that salinity increased protein carbonyl formation (3–6-fold) in *Cicer arietinum* (gram). Enhanced modification of proteins has been reported in plants under various stresses (Sharma et al., 2020). Many other studies have also reported that the enhanced production of ROS during salinity stresses can pose a threat to cells by oxidation of proteins and activation of the Programmed Cell Death (PCD) pathway and ultimately leading to the death of the cells (Møller & Kristensen, 2004; Talaat, 2019).

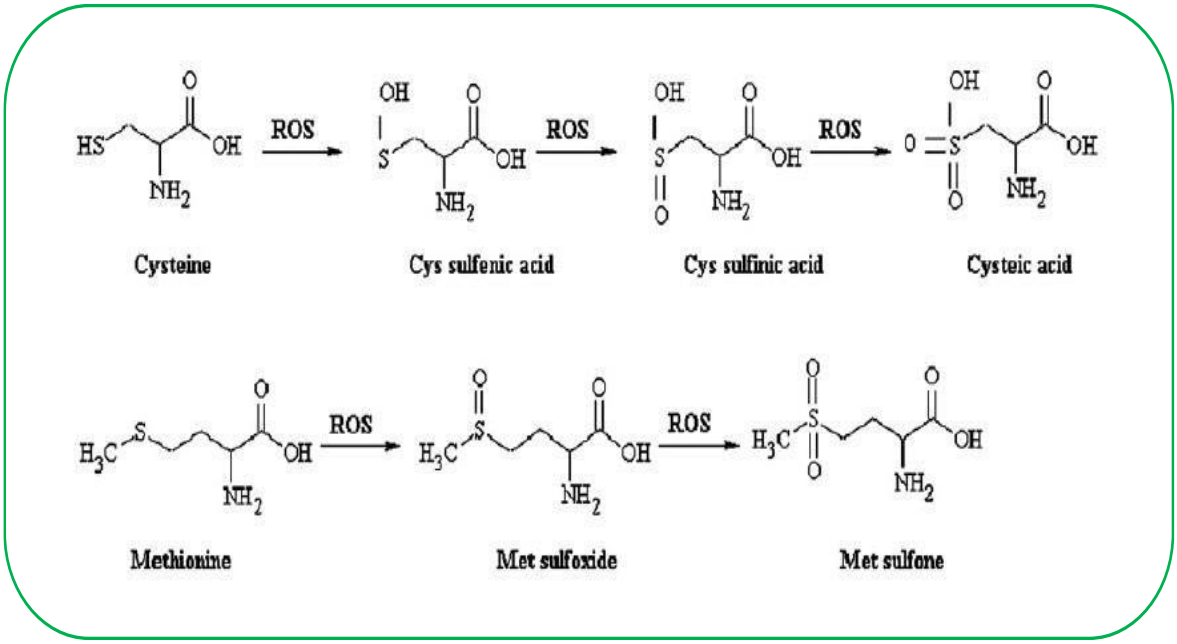


Fig. 1.5.2: Protein oxidation in plants caused by ROS (Gill & Tuteja, 2010).

1.5.3 DNA damage:

DNA is the genetic material of the cells. Any disruption to the DNA can lead to changes in the proteins encoded, leading to malfunctions or complete inactivation of the proteins encoded. The most ROS can cause damage to DNA purine and pyrimidine bases, as well as the deoxyribose backbone. DNA damage occurs via base deletion, cross-links, base modifications (alkylation and oxidation), strand breaks, and pyrimidine dimers resulting in reduced protein synthesis, cell membrane destruction, and damage to photosynthetic proteins. Oxidative DNA damage can affect DNA methylation patterns (Gallo-Franco et al., 2020; Czajka et al., 2022) (Fig.1.5.3). There are reports on salinity-induced DNA damage (Shabala, 2009), genetic modification (Guangyuan et al., 2007), and was reported to be dose-dependent. It was also shown that salinity alters DNA methylation in plants (Dyachenko et al., 2006; Al-Lawati et al., 2016). Excess ROS can damage modified bases and single or double-strand breaks in DNA, and alter cytosine methylation (Konate et al., 2018). The breakage of the DNA with ROS exposure results in impaired protein synthesis; thus, plants fail to evoke its tolerance under such conditions (Sharma et al., 2012; Banerjee et al., 2015).

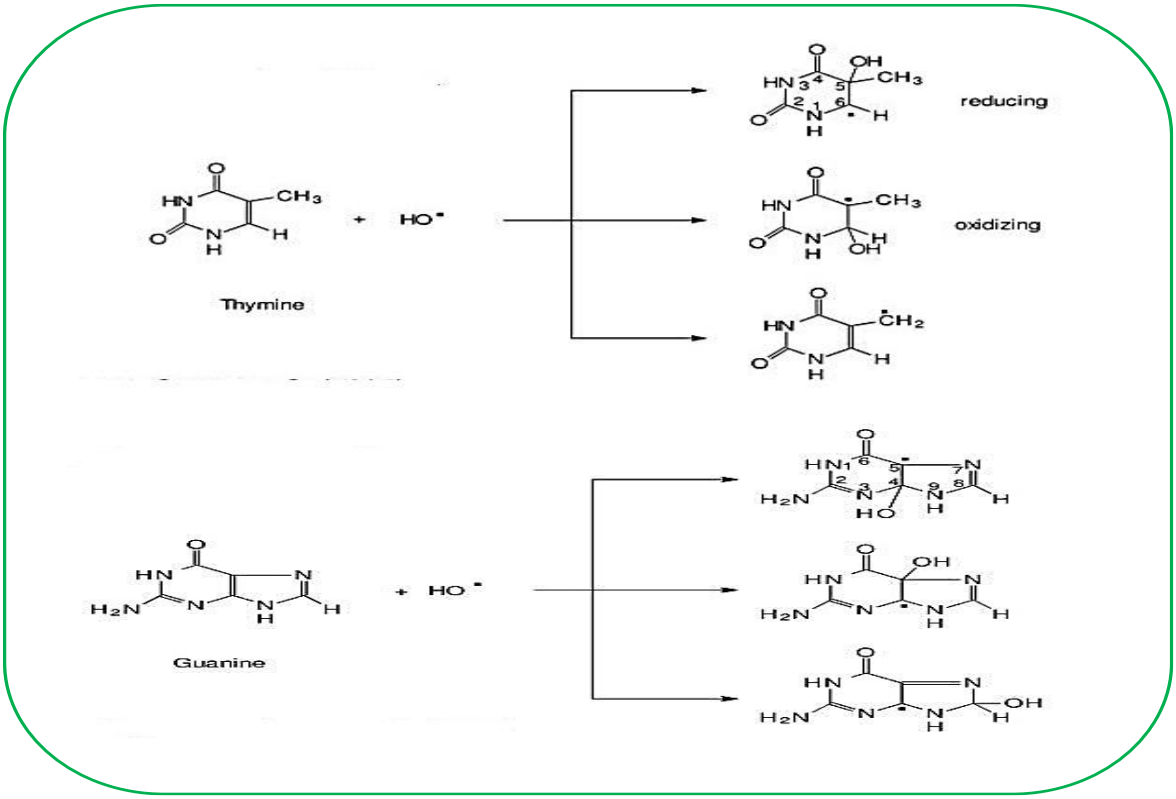


Fig. 1.5.3:DNA oxidation in plants caused by ROS (Sonntag, 1987).

1.5.4 Effect of salt stress on electrolyte leakage

Electrolyte leakage is a hallmark of stress response in intact plant cells. This phenomenon is widely used as a test for the stress-induced injury of plant tissues and a measure of plant stress tolerance (Demidchik et al., 2014). The degree of cell membrane injury induced by salinity stress is easily estimated through electrolyte leakage measurements from the cells (Demidchik et al., 2014; Hniličková et al., 2019). The cell membrane acts as a semi-permeable barrier for the transport of solutes, but the presence of excess salts leads to membrane damage and leakage of ions from the cell (Chawla et al., 2013; Chakradhar et al., 2017; Soltabayeva et al., 2021). It has been demonstrated that electrolyte leakage measurements correlate with several physiological and biochemical parameters such as spectral reflectance (Garty et al., 2000; Bajji et al., 2002), antioxidative enzyme synthesis (Srivastava et al., 2005), membrane acyl lipid concentrations (Lauriano et al., 2000), water use efficiency (Rasool et al., 2013; Garcia et al., 2017), stomatal resistance, osmotic potential, and leaf rolling index (Premachandra et al., 1989). These are mainly caused by the efflux of K^+ and so-called counter ions (Cl^-, HPO_4^{2-}, NO_3^-, citrate^{3-}, malate^{2-}) that move to balance the efflux of potassium ions and recognize that, a range of factors, such as oxidative degradation of the lipid bilayer

(Bajji et al., 2002). Sofy et al. (2020) also stated that electrolyte leakage is mainly related to the efflux of K^+, which is abundant in plant cells. Mineral imbalances in saline environments often affect the structure and chemical composition of the bilayer lipid membrane and may affect the selective ability of the membrane to transport solutes and ions and become leaky (Lodhi et al., 2009; Pavlović et al., 2019). Increased electrolyte leakage under salt stress in an expression of plant organs suffering from toxicity (Feng et al., 2002; Valentovic et al., 2006; Mudgal et al., 2010; Mahlooji et al., 2018; Sarker & Oba, 2020; Kadoglidou et al., 2021). Panda et al. (1996) observed that electron leakage increased in sensitive cultivars than in tolerant ones.

1.6 Salt tolerance mechanisms in plants

Crop performance is severely affected by high salt concentrations, and to engineer more salt-tolerant plants, it is crucial to unravel the critical components of the plant salt-tolerance mechanism.

1.6.1 Role of the antioxidant: Enzymatic and non-enzymatic

It is well known that salinity induces oxidative stress in plants at the subcellular level. The production and removal of ROS to prevent oxidative stress must be strictly regulated. A cell is said to be in a state of "oxidative stress" when the amount of ROS exceeds the defense mechanisms. It is possible to distinguish ROS scavenging mechanisms into two types: enzymatic and non-enzymatic antioxidant defense systems that function to neutralize free radicals synergistically and interactively (Fig. 1.6.1). Such enzymes operate in different subcellular compartments and respond in concert when cells are exposed to oxidative stress. Furthermore, salt-tolerant cultivars possess more enzyme activities than sensitive ones (Islam et al., 2017; Al Kharusi et al., 2019; Roy et al., 2019).

Enzymatic antioxidants:

Enzymatic antioxidants include several ROS scavengers such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX), are primarily involved in enzymatic processes (Apel & Hirt, 2004).

Superoxide Dismutase (SOD): In all aerobic species, SOD plays a central role in protection against oxidative stress (Scandalios, 1993). The SOD enzyme belongs to the metalloenzyme group and catalyzes $O_2^{-\cdot}$ to O_2 and H_2O_2 dismutation. In plants, three

isozymes of SOD are identified copper/zinc SOD (Cu/Zn-SOD), manganese SOD (Mn-SOD), and iron SOD (Fe-SOD) (Racchi et al., 2001). Mn-SOD is localized in mitochondria, while Fe-SOD is localized in chloroplasts (Nath et al., 2014), and Cu/Zn-SOD is localized in cytopsol and chloroplast. In plants exposed to various environmental stresses, including salinity and metal toxicity, SOD activity has been documented to increase (Mishra & Dubey, 2013). SOD is upregulated by abiotic stress conditions (Rasool et al., 2013; Kibria et al., 2017; Lu et al., 2017; Al Kharusi et al., 2019). Up-regulation of antioxidants has been observed in salt-tolerant cultivars of *Pisum sativa*, *Jatropha, Calendula,* and *Oryza sativa* (Gao et al., 2008; Chawala et al., 2013; AbdElgawad et al., 2016), suggesting a pertinent role of antioxidants in alleviating salt stress-induced oxidative damage.

Ascorbate peroxidase (APX): APX is an integral component of the Ascorbate Glutathione (ASC-GSH) cycle. While CAT predominantly scavenges H_2O_2 in the peroxisomes, APX performs the same function in the cytosol and the chloroplast. The APX reduces H_2O_2 to H_2O and DHA, using Ascorbic Acid (AsA) as a reducing agent **(**Rajendra et al., 2019; Abdelaal et al., 2020**).**

$$\mathbf{H_2O_2 + AsA \rightarrow 2H_2O + DHA}$$

The APX family comprises five isoforms based on different amino acids and locations, viz., cytosolic, mitochondrial, peroxisomal, and chloroplastid (stromal and thylakoidal) (Sharma & Dubey, 2007). With an increase in salt-stress, APX activity also increased in wheat (Khan & Patra, 2007; Heidari & Mesri, 2008), cotton (Desingh & Kanagaraj, 2007), *Cakile maritime* (Amor et al., 2006), and in *Broussonetia papyrifera* (Zhang & Shi, 2013). APX activity in the sensitive variety under salt stress showed a negligible increase in activity, whereas the tolerant variety showed a pronounced increase in the activity of APX (Sarker & Oba, 2020). Similar observations were reported by Surekha et al. (2014) in pigeon pea, Al Kharusi et al. (2019) in date palm plants, Chawla et al. (2013) in rice, and Weisany et al. (2012) in soybean and suggested that higher antioxidant enzymes activities could help plants to develop stress tolerance and prevent cell death.

Catalase (CAT): Catalase is a tetrameric heme-containing enzyme responsible for catalyzing the dismutation of H_2O_2 into H_2O and O_2 predominantaly in peroxisome and mitochondria. It has high affinity for H_2O_2 but lesser specificity for organic peroxides (R-O-O-R). It has a very high turnover rate (6×10^6 molecules of H_2O_2 to H_2O and O_2 min^{1}) and is unique among antioxidant enzymes in not requiring a reducing equivalent. Stressful conditions demand greater energy generation and expenditure of the cell. This is fulfilled by increased catabolism which generates H_2O_2. CAT removes the H_2O_2 in an energy-efficient way.

$$\mathbf{H_2O_2 \rightarrow H_2O + (1/2)\ O_2}$$

Rasool et al. (2013) observed that CAT activity increased gradually with salt stress in the tolerant varieties but reduced significantly in the salt-sensitive cultivar of maize. Similar results were observed by Srivastava et al. (2005), the salt-tolerant chickpea, and Gondim et al. (2012) in maize showed significantly higher CAT activity compared to susceptible genotypes at both pre- and post-flowering stages. In salt-tolerant tomato and pea plants, increases in catalase activity have been recorded after a NaCl challenge (Surekha et al., 2014; Acosta et al., 2015). Many workers have reported an increment in CAT activity in salt-tolerant than salt-sensitive plants (Das & Roychoudhury, 2014; Al Kharusi et al., 2019; Sarker & Oba, 2020; Kumar & Hou, 2021). Odjegba and Chukwunwike (2012) in *A. hybridus,* Mohammadi et al. (2018) in sapodilla, Al Kharusi et al. (2019) in Date Palm, and Narimani et al. (2020) in barley reported an increase in CAT under different salt concentrations.

Guaiacol Peroxidase (GPX): GPX, a protein-containing heme, is ideally oxidized at the cost of H_2O_2 by aromatic electron donors such as guaiacol and pyrogallol. These enzymes have four bridges of preserved disulfide and contain two structural ions of Ca^{2+} (Schuller et al., 1996). Many significant biosynthetic processes, including cell wall lignification, IAA degradation, ethylene biosynthesis, wound healing, and defense against abiotic and biotic stresses, are associated with GPX (Kobayashi, 1996). The activity of GPX is enhanced in response to abiotic and biotic stresses, including salinity, heavy metal toxicity, and infection with bacterial or viral pathogens, have been reported by several workers (Avsian-Kretchmer et al., 2004; Ghosh et al., 2014; Srinieng et al., 2015; Naik & Devaraj, 2016). GPXs are commonly known as the “enzyme” of stress. Under stressed conditions, GPX serve as an efficient quencher of reactive intermediate forms of O_2 and

peroxy radicals (Asada, 1992; Apse et al., 1999; Foyer & Noctor, 2003). Tayefi-Nasrabadi et al. (2011) concluded that more excellent protection of salt-tolerant safflower plants from salt-induced oxidative damage, at least in part, was due to the increase of the GPX activity, catalytic efficiency, and induction of specific isoenzymes compared to salt-sensitive cultivar (Sharma & Dubey, 2019**).** Differences in response to salt stress in acclimated and non-acclimated plants suggest a relationship in increased tolerance in acclimated plants and raised activity of GPX (Carrasco-Ríos & Pinto, 2014; Islam et al., 2015; Sofo et al., 2015; Naliwajski & Skłodowska, 2021).

Non-enzymatic antioxidants:

The non-enzymatic antioxidants form the other half of the antioxidant machinery, which includes water-soluble components like ascorbic acid, glutathione, flavonoids, and lipid-soluble components such as carotenoids and α-tocopherol.

Ascorbate (AsA): AsA is the most abundant antioxidant with a low molecular weight that has a key role in protecting against oxidative stress caused by increased ROS due to its capacity to donate electrons in various enzymatic and non-enzymatic reactions. In several physiological processes in plants, including development, growth, differentiation, and metabolism, AsA has been shown to play a significant role. AsA is also synthesized by uronic acid intermediates. D-galacturonic acid is reduced to L-galactonic acid in this pathway by galacturonic acid reductase, which is subsequently converted to L-galactono-1,4-lactone which is oxidized to AsA by the L-galactono-1,4-lactone dehydrogenase (GALDH) enzyme (Upadhyaya et al., 2009). It is synthesized by L-galactono-γ-lactone dehydrogenase in the mitochondria and is transported via a proton-electrochemical gradient or by facilitated diffusion to other cell components. It also acts as a cofactor of violaxanthin de-epoxidase, thus sustaining excess excitation energy (Smirnoff & Wheeler, 2000). Enhancement in ascorbate under saline conditions in salt-tolerant cultivars was also reported in potatoes (Benavides et al., 2000) and beans (Telesinski et al., 2008). A positive correlation between salt tolerance and ascorbate content has been demonstrated in the leaves of sea rockets (Amor et al., 2006) and bitter melon (Agarwal & Shaheen, 2007).

Glutathione (GR): GR, a low molecular weight antioxidant, is a powerful regulator of significant cell functions. Enzymatic antioxidants GSH reductase (GR) detoxify ROS

(Foyer & Noctor, 2003; Manai et al., 2014). GSH serves directly as a free radical scavenger. In the presence of ROS or organic free radicals, GSH can protect macromolecules (i.e., proteins, lipids, DNA) either by the formation of adducts directly with reactive electrophiles (glutathionylation) (Foyer & Noctor, 2003; Rodrigo & Libuy, 2014). It can take part in the regeneration of another potential antioxidant, AsA, via the AsA-GSH cycle. GSH recycles AsA from its oxidized to reduced form by the enzyme DHAR (Loewus, 1988; Ali & Ashraf, 2011). Researchers have obtained elevated GR activity in *Calamus tenuis* (Khan & Patra, 2007) and *Phaseolus vulgaris* (Nagesh Babu & Devaraj, 2008) in response to salt tolerance. Similarly, higher GR activity in salt-tolerant cultivars and diminished activity in the sensitive cultivars was reported in wheat (Mandhania et al., 2006) and maize (Kthiri et al., 2022).

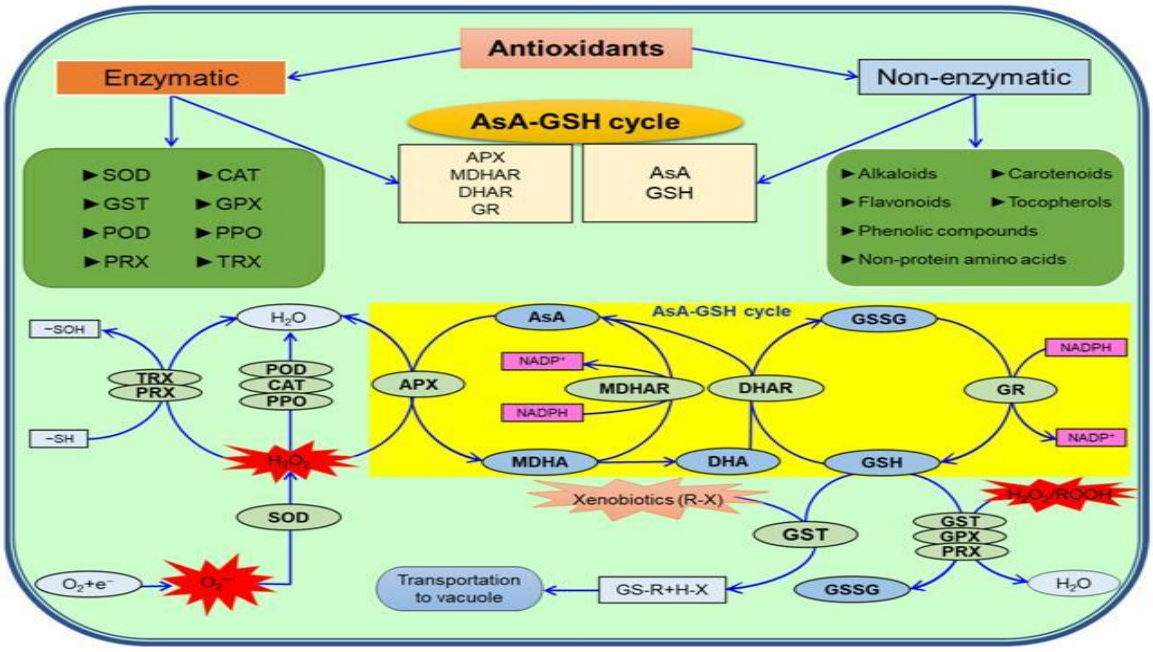


Fig. 1.6.1: Overview of different types of antioxidants and their combined mechanisms. DHA-dehydroascorbate; GSSG-oxidized glutathione; GSH-reduced glutathione; APX-ascorbate peroxidase; MDHA-monodehydroascorbate; MDHAR-monodehydroascorbate reductase; DHAR-dehydroascorbate reductase; GR-glutathione reductase; GST-glutathione S-transferase; GPX-glutathione peroxidase; PPO-polyphenol oxidase; PRX-peroxiredoxins; TRX-thioredoxin; NADPH-nicotinamide adenine dinucleotide phosphate; O_2-oxygen; e^--electrons; R-aliphatic, aromatic or heterocyclic group; X-sulfate, nitrite or halide group; ROOH-hydroperoxides; -SH-thiolate; -SOH-sulfenic acid (Hasanuzzaman et al., 2020).

Tocopherols: Tocopherols (alpha, β, γ, and δ) are a category of lipophilic antioxidants to oxygen-free radicals, lipid peroxide radicals, and $^1O_2$ radicals (Rice-Evans et al., 1989). The relative antioxidant activity of tocopherol isomers is α > β > γ > δ due to the pattern of methylation and the number of methyl groups attached to the polar head structure phenolic ring (Rasool et al., 2013). Tocopherols are only synthesized by photosynthetic species and are only found in parts of green plants. Generally, α-tocopherol accumulates mainly in leaves, seeds are rich in γ-tocopherol but β-tocopherol and δ-tocopherol are not very abundant in most plant species. NaCl is reported to increase α-tocopherol content significantly (Chen et al., 2018). Two compounds, Homogentisic Acid (HGA) and Phytyl Diphosphate (PDP), are used as precursors in the tocopherol biosynthetic pathway. Tocopherols as antioxidants alleviate oxidative damage leading to improvements in physiological attributes in plants grown under the adverse conditions of saline soils (Semida et al., 2016). Tocopherols prevent lipid auto-oxidation from the chain propagation stage, making it an efficient free radical trap, preserving PSII from photo-inactivation, and protecting membrane lipids from photo-oxidation (Lalarukh et al., 2022).

1.6.2 Ion regulation and compartmentalization

Maintaining ion homeostasis by ion exclusion and compartmentalization is crucial for normal plant growth and an essential process for growth during salt stress. Ion regulation and compartmentalization is one method used by both glycophytes and halophytes (Parida & Das, 2005).

Sodium is a functional nutrient in plants involved in different metabolic functions due to the parallelism between sodium and potassium. These functions include being a cofactor in enzyme activation, stabilizing the active conformation of enzymes and possible membranes, cytoplasmic volume regulation, energy conservation across membranes, and the regulation of cytoplasmic pH (Pessarakli, 2014). Nevertheless, an increase in sodium concentration can destabilize membranes and proteins, negatively affecting fundamental processes like the division and expansion in a cell, primary and secondary metabolism, and the homeostasis of mineral nutrients (Hasegawa, 2013). Plant cells are responsible for reducing and accumulating ion concentrations at adequate levels to regulate the ion flux to achieve this homeostasis.

It is well known that Na^+ relocation from the root zone to the other organs of the plants takes place by flow mass controlled mainly by the pressure gradient (Taiz & Zeiger,

2010). Thereupon, the movement of Na^+ from the root zone to the xylem takes place via symplastic, apoplastic, or intercellular spaces until the endodermis with the Casparian strip, which limits the apoplastic movement (Craig Plett & Møller, 2010) (Fig. 1.6.2). Regulation of the expression and the activity of K^+ and Na^+ transporters and H^+ pumps are important for ion compartmentalization as they help to maintain high levels of K^+ and low levels of Na^+ in the cytosol under salt stress (Schroeder et al., 2013).

K^+ and Na^+ compete for the same binding sites, so if there is a depolarization in the membrane by an increase of Na^+ concentration, the main consequence will be a decrease of K^+ uptake and an increase of K^+ efflux through outward-rectifying channels (Adem et al., 2014). The maintenance of high K^+ levels and low Na^+ in the cytosol of a cell is controlled by H^+-ATPase (active transport) and channels and co-transporters (secondary transport). One way to reduce the concentration of Na^+ in a cell is the exclusion and its compartmentation in the vacuole; therefore, this mechanism has a high significance in salt tolerance at the cellular level (Deinlein et al., 2014). However, Na^+ transporters are involved in the extrusion of Na^+ from cells at the plasma membrane via Na^+/H^+ antiport, sequestration of Na^+ into plant vacuoles, and blockage of Na^+ over-accumulation in leaves (Shi et al., 2002; Zörb et al., 2005). A tonoplast-localized Na^+/H^+ exchanger (NHX1) and a plasma membrane-localized Salt Overly Sensitive 1 (SOS1/NHX7) are two major Na^+ transporters, important for the salt stress resistance mechanisms in plants (Brini & Masmoudi, 2012; Osakabe et al., 2014). AtNHX1 over-expression studies by Apse et al. (1999) and Zhang and Blumwald (2001) showed that NHX1 played a major role in salt tolerance in transgenic plants, *A. thaliana*, *Solanum lycopersicum*, and *Oryza sativa*. Six intracellular NHX-type antiporters were found in *A. thaliana*, which can be classified into two groups (Sánchez-Rodríguez et al., 2010; Barragán et al., 2012). NHX1 to NHX4, antiporters associated with vacuoles, belong to Group 1, whereas NHX5 and NHX6, associated with endosomal components, belong to Group 2 (Bassil & Blumwald, 2014). NHX1 is involved in Na^+ detoxification by removing excess Na^+ from the cytosol and compartmentalization in the vacuoles, whereas SOS1 is important to export Na^+ out of the cells (Deinlein et al., 2014). Na^+/H^+ antiporter, SOS1 is regulated by the SOS2 Ser/Thr protein kinase and two calcium sensors, SOS3/CBL4 (Calcineurin B-like 4) and SCaBP8/CBL10 (SOS3 homolog SOS3-Like Calcium Binding Protein8/ Calcineurin B-like 10) (Quan et al., 2007). This SOS2-SOS3 complex phosphorylates and activates the SOS1 transporter, which extrudes excess Na^+ from the cytosol (Qiu et al., 2003; Quintero et al., 2011). Shi et al. (2002) showed that SOS1 is expressed in the epidermis of the root

tip region and xylem parenchyma cells for partition of Na^+ between roots and shoots (Ali et al., 2021). SOS1 is involved in Na^+ efflux at the root epidermis and is indirectly important for K^+ uptake in the cells (Huang et al., 2012). It is also responsible for the ion concentration of cells by controlling Na^+ loading and unloading from xylem vessels and long-distance Na^+ transport from roots to shoots (Olías et al., 2009). Apart from salt tolerance, NHX antiporters are known to be involved in flower coloration, K^+ homeostasis, cell expansion, vesicular trafficking, and protein targeting (Apse & Blumwald, 2007; Lodhi et al., 2009; Barragán et al., 2012).

The current model of the SOS pathway explains that an increase in the intracellular Ca^{2+} due to high Na^+ concentrations encourage the Ca^{2+} binding to SOS3, which interacts with and activates SOS2. SOS2 and SOS3 physically interact and form the SOS2-SOS3 complex, where activated SOS2 phosphorylates the plasma membrane-localized SOS1. Phosphorylated SOS1 increases the Na^+ efflux under salt stress (Zhang & Shi, 2013). A study by Feki et al. (2014) observed that over-expression of a truncated form of wheat SOS1 (TdSOS1, deletion of the auto-inhibitory domain) in *A. thaliana* enhanced root elongation, water retention, and salt tolerance. A transporter called NHX1 belongs to the CPA1 family (a monovalent cation/proton antiporter family) (Mäser et al., 2002) and is found on the plasma membrane, in endosomal compartments, and in vacuoles (Apse & Blumwald, 2007; Hamaji et al., 2009; Barragán et al., 2012).

Studies pointed out that the activity of SOS1 coordinates with High-Affinity Potassium transporter gene (HKT) activity in the plasma membrane of xylem parenchyma cells to achieve adequate. HKT1 proteins are essential as they are involved in the Na^+ ascending from xylem sap and recirculating Na^+ from leaves to roots, thus restricting the amount of Na^+ reaching the photosynthetic tissues (Brini & Masmoudi, 2012; Deinlein et al., 2014) (Fig. 1.6.3). The HKT gene is classified into two groups according to their amino acid sequence. Class I consist of uniporter (Na^+ selective) having a serine at the first pore domain, which includes (OsHKT1;1) (Jiang et al., 2018), and group II is OsHKT2;4 (K^+/Na^+ symporter) (Mishra et al., 2016). Sodium reabsorption at xylem parenchyma cells mediated by HKT transporters has appeared as an essential factor for plants to maintain a high K^+/Na^+ ratio in the cytosol, which mediate Na^+ specific transport or Na^+/K^+ transport and play a key role in the regulation of Na^+ homeostasis which confers salt tolerance (Munns & Tester, 2008; Fraile-Escanciano et al., 2010; Hauser & Horie, 2010; Hamamoto et al., 2015). OsHKT1:1 overexpression promoted shoot Na^+ accumulation under low K^+ supply and proposed that OsHKT1:1 expression level is a

critical factor in the Na^+ accumulation potential in rice varieties (Miyamoto et al., 2015). OsHKT2;4 expressions contribute to a lower cytosolic Na^+/K^+ ratio (Hauser & Horie, 2010). Studies on AtHKT1;1 and its rice homolog OsHKT1;5 showed that the Na^+ transporter helped to protect photosynthetic tissues by removing excess Na^+ from xylem sap into surrounding xylem parenchyma cells (Davenport et al., 2007).

Chloride is an essential micronutrient responsible for synchronization of enzymatic activities in the cytoplasm, a cofactor in photosynthesis, involvement in pH regulation, and regulation of membrane potential and turgor through the counteraction of anions (Teakle & Tyerman, 2010; Li et al., 2017). The toxicity threshold of Cl^- is estimated to be 15-50 mg g^{-1} for Cl^- tolerant and 4-7 mg g^{-1} dry weight for sensitive species (Xu et al., 1999). Visual Cl^- toxicity symptoms often start with chlorotic discolorations that turn into necrotic lesions, resulting in the symptom of leaf-tip burning. A higher concentration of Cl^- inhibits the photosynthetic rate by reducing the root nitrate uptake resulting in reduced growth and yield (Tavakkoli et al., 2010). The movement of Cl^- transport through the cell membrane can be performed in two ways: a $2H^+/Cl^-$ symporter, and the antiport using hydroxyl ions activated by ATP. Compartmentalization of Cl^- ions through ion channels is an important stress tolerance mechanism in plants (Brini & Masmoudi, 2012). Voltage-gated ion channels belonging to the CLC (chloride channel) family are known to be involved in vacuolar Cl^- sequestration (Brini & Masmoudi, 2012; Kaur & Pati, 2017). A study by Diédhiou and Golldack (2006) showed the importance of OsCLCc in the osmotic adjustment of salt-treated rice plants. Lower xylem Cl^- was found in relatively salt-tolerant varieties compared to salt-sensitive varieties of wheat (Läuchli et al., 2008) and *Lotus corniculatus* (Teakle et al., 2010).

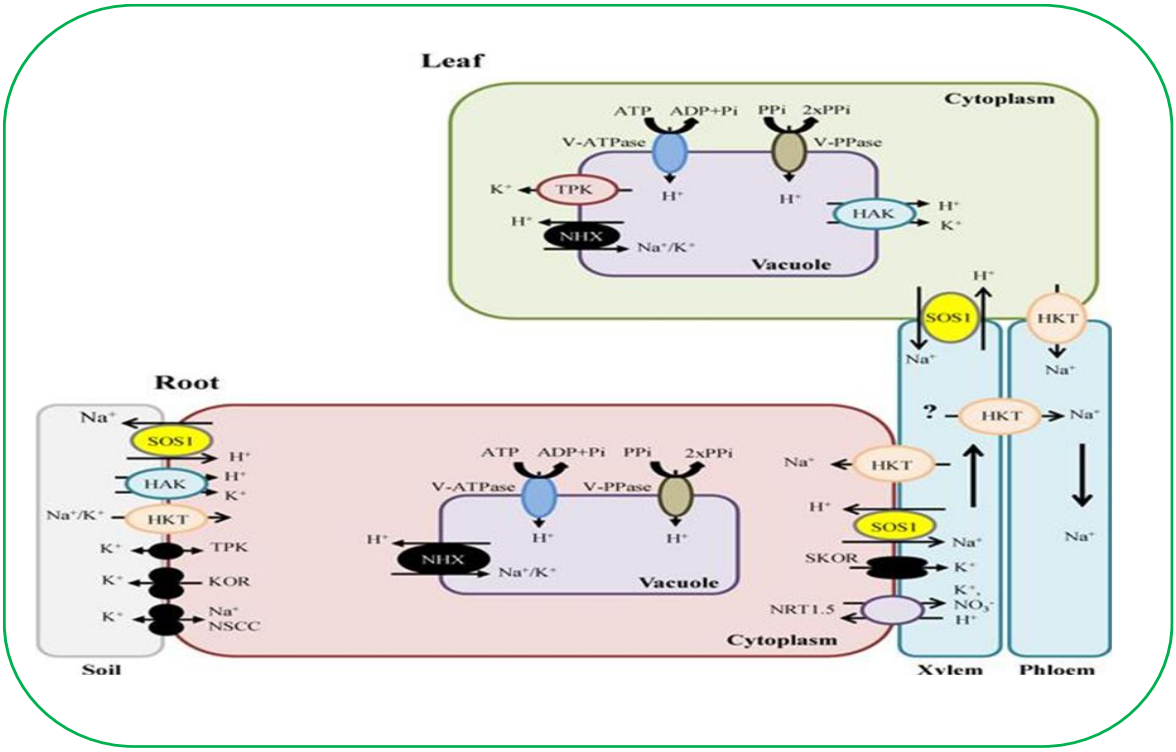


Fig. 1.6.2: Schematic representation showing Na^+ ions enter the cells via Non-Selective Cation Channels (NSCCs) and possibly via other cation transporters (symplast flow) and through the cell wall and intercellular spaces (apoplast flow). The Na^+/H^+ antiporter SOS1 extrudes Na^+ at the root-soil interface, thus reducing the Na^+ net influx of Na^+. In addition, HKT1-like proteins also load Na^+ into the shoot phloem, then Na^+ is transferred into roots via the phloem, preventing Na^+ accumulation in shoots. SOS1, localized in the xylem parenchyma cells, is also suggested to mediate Na^+ efflux from xylem vessels under high salinity. Incoming Na^+, in root and shoots, is stored in the large central vacuole by tonoplast localized NHX exchangers. Plasma membrane (PM) H^+-ATPase (P-ATPase), PM H^+-PPase (PM-PPase), tonoplast H^+-ATPase (V-ATPase), and tonoplast H^+-PPase (V-PPase) generate electrochemical potential gradient for secondary active transport (Saibi & Brini, 2021).

1.6.3 Compatible solutes

The accumulation of osmolytes is another protective mechanism of plants that help in survival under salt stress by maintaing water level (Hasegawa et al., 2000; Parida & Das, 2005) (Fig. 1.6.3). The organic osmolytes such as Proline (Pro) and Glycine-Betaine (GB) are the most significant and well-organized compatible solutes (Anjum et al., 2016; Ayub et al., 2022) and confer plant tolerance to salt stress (Zengin, 2015; Sharma et al., 2019). In plants, Pro synthesis is mainly catalyzed by Δ-1-pyrroline-5-carboxylate synthetase

(P5CS), which converts glutamate into Δ-1- pyrroline-5-carboxylate (P5C) in the cytoplasm of the chloroplast. P5C reductase (P5CR) promotes and reduces P5C to Pro (Hare & Cress, 1997). Pro degradation, taking place in the mitochondria, is mediated by proline dehydrogenase (ProDH), which produces P5C from proline, and P5C dehydrogenase, which converts P5C to glutamate (Cecchini et al., 2011). These osmoprotectants lower the water potential to maintain better plant water status to enhance plant productivity, and inducing tolerance in response to salt stress.

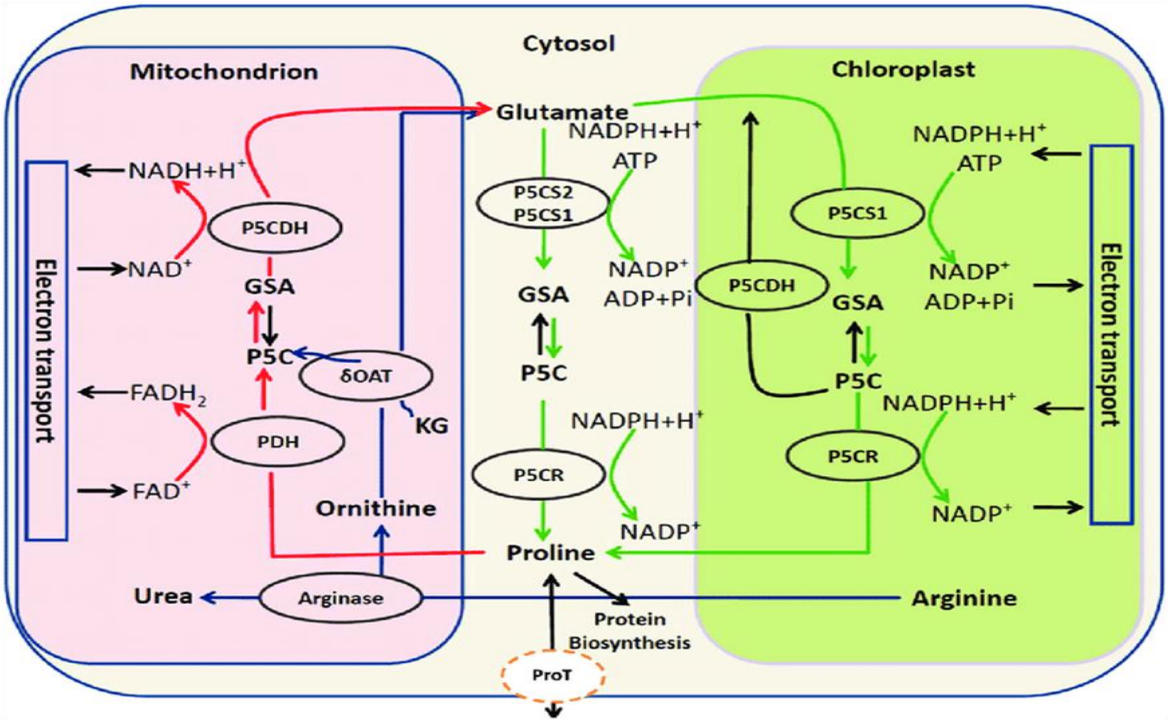


Fig. 1.6.3: Schematic representation of proline metabolism in plants: Proline synthesis occurs in the cytosol and chloroplast. Proline degradation occurs in mitochondria. P5C - δ-pyrroline-5-carboxylate, P5CR -pyrroline-5-carboxylate reductase, P5CS-pyrroline-5-carboxylate synthase, GSA-glutamic semialdehyde, PDH -Proline dehydrogenase, OAT -Ornithine aminotransferase, KG-Ketoglutarate, Port-Proline transporter (Khanna-Chopra et al., 2019).

Many studies suggested that salt stress modulates the enzymes responsible for Pro and GB (Sumithra et al., 2006) and their increased concentration is correlated with increased stress tolerance (Khedr et al., 2003; Hasanuzzaman et al., 2013). In general, salt-tolerant species accumulate Pro after a salt-stress challenge. For example, *Eugenia myrtifolia L.* plants showed a salt-tolerant response when treated with 8 dS m^{-1} NaCl for 30 days; as

observed by plant growth, this response correlated with a 20% increase in leaf proline contents (Acosta et al., 2017). The Pro content increased with the increase in the duration of stress (Conde et al., 2011; Chutipaijit et al., 2011).

1.7 Omics-based approaches to improve salinity tolerance

Plant molecular biology attempts to research biological and cellular processes, such as plant growth, the structure of their genomes, and contact with their environment. These multidimensional, comprehensive studies involve large-scale experiments connecting genetic, functional, and structural components. These large-scale studies are referred to as 'omics'. It includes genomics, transcriptomics, proteomics, metabolomics, and phenomics (Das et al., 2015). Changes in the metabolome composition due to adverse environmental conditions may depend on the stability and catalytic activity of enzymes involved in the production/degradation of specific metabolites, ii) the production of abnormal compounds (or abnormal concentrations of normal compounds) as a result of cell damage, iii) the adjustment of the concentration of some metabolites to restore homeostasis and normal metabolic fluxes and iv) the synthesis and accumulation of compounds involved in mediating tolerance mechanisms. High throughput LC-MS approaches enable the rapid identification of large sets of proteins and their post-translational modifications (Wang et al., 2020). Plant acclimation to abiotic stress conditions is associated with profound changes in proteome composition and since they are directly involved in plant stress response, proteomics studies can significantly contribute to unraveling the possible relationships between protein abundance and plant stress acclimation (Genga et al., 2011).

Transcriptomics, also called expression profiling, typically involves all RNA transcripts systematically and thoroughly analyzed, meaning the spatial and temporal gene expression of an organism cell and the tissue under a specific biological situation (Thompson & Goggin, 2006). The mechanisms involved in salinity tolerance are complex and polygenic features are well known (Munns & Tester, 2008). In this regard, transcription variables with a cascade effect that can influence several other downstream genes can also prove essential. Salt tolerance in rice is advantageous as rice is significantly salt-sensitive to the seedling and reproductive phase, and a few QTLs having significant effects are known to control the trait (Leung, 2008). However, the markers have low heritability and are typically quantitatively inherited (Cuartero et al., 2006). The

transcriptomic approach provides an efficient method to recognize the gene(s) involved in particular stress, in addition to the challenge of identifying the related target genes.

1.8 Rice cultivation in India

Rice (*Oryza sativa* L.) is a cereal food crop that belongs to the plant kingdom's grass family Poaceae. Rice is native to tropical and subtropical southern Asia and southeastern Africa (Crawford & Shen, 1998). Although it can grow in diverse environments, it grows faster and more vigorously in wet and warm conditions. This plant develops a main stem and many tillers and may range from 0.6 to 6 m (floating rice) in height. The tiller bears a ramified panicle between 20 and 30 cm wide. Each panicle has 50–300 flowers (floret or spikelet), which form the grains, and the fruit obtained is a caryopsis (Agropedia). Rice is one of the most important food crops and feeds more than 60 percent population of India. The area under rice crop was 30.81 million ha^{-1} in 1950-51, which has increased to 43.86 million ha^{-1} during 2014-15, which is nearly 142% higher. Rice production has registered an appreciable increase from 20.58 million tonnes in 1950-51 to 104.86 million tonnes from 2014-15, nearly 5 times. The yield was 668 kg ha^{-1} in 1950-51, which has increased to 2390 kg ha^{-1} during 2014-15. Estimates suggest global economic losses due to soil salinization are around US $ 27.3 billion per year (Wichelns & Qadir, 2015). The major share of rice production is in the Kharif season. The area, production, and productivity of the first five highest-producing countries of rice in the world are given below (Table 1.8.1).

Table. 1.8.1: Country, Area, production, and yield of rice.

Sr.No.	Country	Area, million ha	Production, million tonnes	Productivity, kg/ha	Production %
1	India	43.86	104.80	6710	27.70
2	China	30.58	205.21	2390	21.81
3	Indonesia	13.84	71.29	5152	9.62
4	Bangladesh	11.78	51.50	4376	6.95
5	Vietnam	7.90	44.04	5573	5.94
6	Total the world	107.96	476.84	4417	-

Source. Agricultural statics at a glance 2015.

1.8.1 Region-wise rice ecosystems in Goa

Agriculture is one of the important economic activities of Goa state, located between the Arabian Sea and the Western Ghats, Goa faces problems with enough cultivable land to feed its population. The coastal areas are exposed to salinity and do not qualify as the right agricultural areas, while the inland areas are low in productive. Rice and fish being the staple diet of the people, paddy is the principal crop in Goa (UKEssays, 2018). The yield of rice crops in salt-affected coastal soils of Goa is low (1.5 to 2.0 tonnes ha^{-1}) due to high salinity levels, especially during the seedling and maturity stages. Country's total rice-cropped area, about 55% is occupied in the coastal regions (Rai, 2004).

The total rice area of 49,966 ha in Goa State, North Goa, comprising six talukas viz., Tiswadi, Bardez, Pernem, Bicholim, Sattari, and Ponda, covers an area of 28,119 ha, accounting for 56.3% of the area, while the rest is covered by the South Goa, comprising five talukas, viz., Sanguem, Canacona, Quepem Salcete, and Murmagoa. While upland rice cultivation dominates the rice ecosystem in talukas adjacent to the Western Ghats, the lowland rice and the salt-tolerant rice (in the grassed area of seawater all along the seacoast) dominate the coastal ecosystem.

'Korgut,' a low-yielding but salt-tolerant variety of southern India, is grown in marshy areas close to the sea, often in conjunction with prawn farming. On the other hand, 'Jaya' is a high-yielding, sensitive variety grown inland in southern India. 'Korgut' yields 2 to 2.5 tons ha^{-1} and 'Jaya' yield between 4-4.5 tonnes ha^{-1}. Importance of rice as a staple food and the damaging effects of salinity on this crop species, we designed the present study mainly intending to assess salinity-induced changes concerning growth, morphology, physiological, biochemical, and molecular processes in both, sensitive and tolerant, varieties. A comparison of these responses could help identify the strategies which make 'Korgut' salt-tolerant, and such properties can be imparted in developing rice varieties through genetic engineering or conventional plant breeding to develop high-yielding but salt-tolerant varieties in the future. The study will help our understanding of salt tolerance in rice.

1.9 Aim and Objectives

Asia is known as the world's primary rice producer by yielding more than 650 million tons (90% of total rice yield worldwide) grown in 145 million ha of land. Crop productivity is severely affected by salinity stress. This occurs directly due to salinity's

impact on photosynthesis, respiration, nutrient assimilation, hormonal imbalance, etc. Because of the importance of rice as a staple food and the damaging effects of salinity on this crop species, we designed the present study. 'Korgut,' a tolerant variety of southern India, is grown in marshy areas close to the sea, often in conjunction with prawn farming. On the other hand, 'Jaya' a high-yielding, relatively sensitive variety grown inland in southern India. Comparing salt-sensitive and tolerant rice varieties to investigate the relationships between morpho-physiological, biochemical, and molecular characteristics may help select the traits to develop rice cultivars suitable to grow in coastal soil.The objectives set out were;

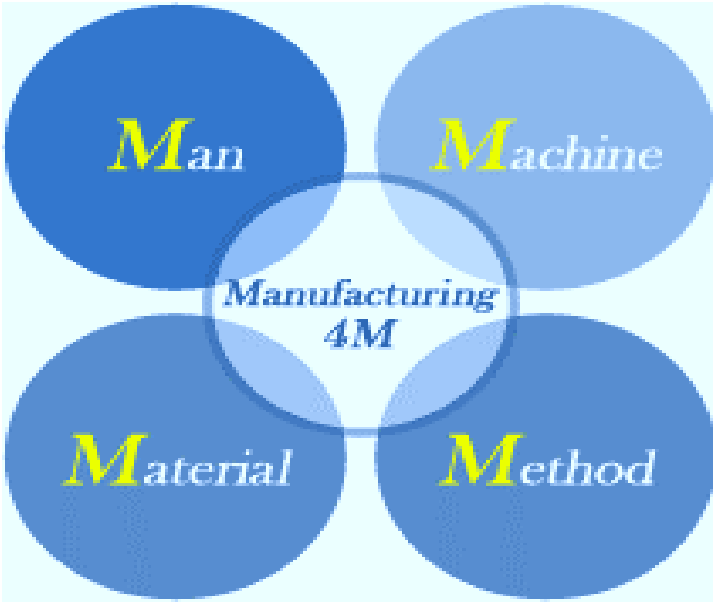
Objective 1. To study the effect of NaCl concentration on growth, photosynthesis, and pigments.

Objective 2. To study the effect of NaCl on biochemical parameters.

Objective 3. To study the accumulation of NaCl in the leaves and roots tissue.

Objective 4. To study the effect of NaCl on protective processes of biochemical and molecular.

CHAPTER 2



MATERIALS AND METHODS

“Learning never exhausts the mind.”

Leonardo da Vinci

2.1 Plant materials and growth conditions

Seeds of 'Jaya,' high-yielding salt-sensitive rice (*Oryza sativa*) variety, and 'Korgut' (INGR14055), a 100% salt-tolerant rice variety, were obtained from the Indian Council of Agricultural Research – Central Coastal Agricultural Research Institute, Goa and were stored in the dark at 4ºC. NaCl (Merck, A.R. Grade) solutions of 0 (control, without NaCl treatment), 40, 80, 120, 160, and 200 mmol/l were prepared in Hoagland's solution (pH 6.5). Seeds were surface sterilized with 4% sodium hypochlorite solution (NaClO, Merck, A.R. Grade), washed thoroughly with distilled water, and soaked in fresh water for 4 days. Plastic pots of 20 cm diameter filled with vermiculite were used for plant growth, and approximately 100 seeds were sown in each pot with a particular concentration of NaCl. This technique controls soil heterogeneity by using vermiculite as a growth medium. Plants were grown for 21 days in growth chambers illuminated with fluorescent and incandescent lamps with a 16 h photoperiod of 200 µmol m^{-2} s^{-1} Photosynthetically Active Radiation (PAR), and the temperature was maintained at 26 ± 2ºC with a relative humidity of 70-75%. Each pot was considered as one replicate with three pots per treatment per variety. The experimental design was done using the randomized block method.

2.2 Growth measurements and biomass studies

Root and shoot lengths were manually measured from randomly selected plants after 21 days of growth. The shoot and root biomass were calculated by taking ten plants from each treatment group after 21 days of growth and weighed for the Fresh Weight (FW). The tissue was then dried at 60ºC for 72 h, and the Dry Weight (DW) was measured.

2.3 Relative water content (RWC)

The leaf Relative Water Content (RWC) was assessed just after collecting plants. Fresh weight (FW) was taken without delay, and then leaves were rehydrated by floating them in distilled water with a few drops of Tween-20 at room temperature for 4 h. After rehydration, leaf turgid weight (TW) was measured, and then the leaf was kept in an oven at 60°C for 72 h. After 72 h, the leaf dry weight (DW) was measured, and leaf RWC was calculated using the formula (Barrs, 1968; Boyer, 1968).

$$\textbf{RWC \% = (FW-DW/TW-DW) *100}$$

Table 2.1: Composition of Hogland's plant nutrient solution (Hoagland & Arnon, 1950).

Sr. No.	Chemical composition	Stock Solution (g/L)	From stock solution mL /1L
1	2M KNO_3	202 g/l	2.5
2	2M Ca $(NO_3)_2$ •4H2O	236 g/0.5 L	2.5
3	2M $MgSO_4$ •$7H_2O$	493 g/l	1
4	1M KH_2PO_4 (pH 6.0)	136 g/l	0.5
5	1M NH_4NO_3	80 g/l	1
6	H_3BO_3	2.86 g/l	1
7	$MnCl_2$ •$4H_2O$	1.81 g/l	1
8	$ZnSO_4$ •$7H_2O$	0.22 g/l	1
9	$CuSO_4$	0.051 g/l	1
10	Na_2MoO_4 •$2H_2O$	0.12 g/l	1
11	$C_{12}H_{12}Fe_2O_{18}$	15 g/l	1

2.4 Morphological studies

2.4.1 External morphological study (SEM)

The external morphological studies to observe the variation in size, shape, and number of stomata and trichomes were carried out using SEM. The middle region of the leaf was cut into small pieces of approximately 1.5 x 1.5 mm in dimensions. The treated samples were stored at 20ºC for 24 h in glass beakers and then allowed to reach a temperature of -110ºC using a Cool Safe 110 Freeze Dryer (Fisher Scientific Bio-block) for 30 min under vaccum.The samples were allowed to undergo sublimation for 2 h with the external valve closed. The vacuum pump was allowed to run for 30 min by keeping the external valve closed. These samples were coated with gold using a fine auto coater (JEOL JFC-1600, Japan) for 20 min. Coated samples were visualized using (SEM) Scanning electron microscopy (JSM 5800 LV, JEOL, Japan).

2.4.2 Internal morphological (Anatomy) study (TEM)

Internal leaf morphology was analyzed using Transmission electron microscopy (TEM) at the All-India Institute of Medical Sciences (AIIMS), New Delhi. Fresh leaf tissue was cut into 2 × 2 mm dimensions and washed with 0.1% Tween-20 for 2 min, then rinsed with distilled water twice. Sample infiltration was carried out using a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 10 h at 4°C. The tissue sample was washed in 0.1 M chilled phosphate buffer and fixed at room temperature for one h in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.2). The sample was dehydrated in 100% acetone, infiltrated, and embedded in Araldite CY 212. Thin serial sections (70-80 nm thick) were obtained on copper grids using ultra-microtome (Leica UC6, Leica Microsystems, Germany) stained with uranyl acetate and lead citrate, and examined using a TEM (Morgagni 268D, Fei Company, The Netherlands) according to Da Costa and Sharma (2016).

2.5 Uptake studies of Na^+, K^+, and Cl^-

2.5.1 Estimation of Na^+ and K^+ in leaf and root tissue

The Na^+ and K^+ concentrations in leaf and root tissue were carried out according to Yoshida et al. (1976) using a Digital flame photometer (LT-65, LT-66, Labtronics, India). The tissue (0.5 g) was dried, powdered, transferred to crucibles, and placed in a muffle furnace at 450ºC for 5 hours. The ash obtained was digested in 1N HCl for 48 h. The sample concentration was calculated using the standard series prepared from 1000 ppm of sodium chloride from the stock solution.

2.5.2 Estimation of Cl^- in leaf and root tissue

Cl^- content in leaf was determined according according to Teakle and Tyerman (2010). Fresh leaf and root tissue (0.2 g) were lyophilized at -120ºC for 5 h. Deionized water and glacial acetic acid were added, kept on the oscillating shaker for 25 min, and later centrifuged at 5000 rpm for 15 min. The extracted solutions was analyzed for ionic concentration using Ion exchange chromatography (919 IC Autosampler plus Swiss model, Metrohm) and analyzed using IC magic net 2.4 workshops. Sample concentration was calculated using the standard series prepared from 100 ppm of chloride stock solution (Chloride ISE 100 ppm).

2.6 Photosynthetic measurements

Various parameters of photosynthesis, such as CO_2 fixation, transpiration rate, stomatal conductance, chlorophyll fluorescence, and pigments were measured.

2.6.1 Gas Exchange measurements

Net photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (gs) internal carbon were performed using a portable infra-red gas analyzer (IRGA, ADC Bio scientific, LCi-SD, Hansatech, UK) according to Sharma and Hall (1996) at light intensity of 1200 µmol m^{-2} s^{-1} using a detachable light source provided by a diachronic lamp (Hansatech, UK). The leaf sample parameters to be analyzed are enclosed in the leaf chamber consisting of a handle (PCA-275) with IR-red source assembly and a tube set. The IR assembly detects the gas in the leaf sample. A silicon-based sensor measures PAR, chamber temperature, and leaf temperature are monitored by a thermistor sensor, and gas flow rate (U) to the chamber is measured by a mass flow sensor. From the controlled mass flow rate (Uset) and the differences in the reference and analyzed gases, net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and internal CO_2 concentration (Ci) are calculated. All measurements were made at ambient temperature and CO_2 according to Sharma and Hall (1996), and the values of the parameters were calculated according to Long and Hallgren (1993).

Calculate assimilation rate, P_N (mol m^{-2} s^{-1})

$$P_N = \frac{f}{s} \bullet \Delta c, \quad \Delta c = \frac{(eo - ei)}{(1 - eo)}$$

Calculate transpiration rate, E (mol m^{-2} s^{-1})

$$E = \frac{f}{s} \bullet \frac{(eo - ei)}{(1 - eo)}$$

Calculate stomatal conductance, gs (mol m^{-2} s^{-1})

$$gs = \frac{E}{es,T1 - eo}$$

Calculate the internal CO_2 concentration, Ci

$$Ci = C_0 - \frac{\mathbf{PN \times 1.6}}{\mathbf{gs}}$$

Where, s = leaf area surface m^2

f = mole flow of air (mol s^{-1})

Δc = CO_2 differntial between reference and analysis streams (mol mol^{-1})

e_0 = mole fraction of water vapour at leaf chamber outlet (mol mol^{-1})

ei = mole fraction of water vapour at leaf chamber inlet (mol mol^{-1})

es = mole fraction of water vapour at saturation.

T1=Leaf saturated with water vapour at the actual leaf tempereture

C_0 = mole fraction of CO_2 in outlet air from leaf chamber (µmol mol^{-1})

Ci = mole fraction of CO_2 in inlet air from environment of leaf chamber (µmol mol^{-1})

2.6.2 Fluorescence measurements

Chlorophyll fluorescence parameters, minimal fluorescence (Fo), maximal fluorescence (Fm), variable fluorescence (Fv), and the ratio Fv/Fm of fully expanded 1 cm^2 per cultivar per treatment combination was measured *in vivo*, 30 min of dark adaptation of the leaves was carried out using a fluorescence monitoring system (PAM instruments) according to Sharma and Hall (1996). The dark-adapted leaf was manifested to a weak modulated light 3 µmol m^{-2} s^{-1} to measure initial fluorescence (Fo). Afterward, exposure to a saturating pulse of the white light of 4000 µmol m^{-2} s^{-1} provides the maximum fluorescence (Fm). Subsequently, measurement of Fm, the leaf was allowed to reach steady-state fluorescence (Fs) by exposing it to the actinic light intensity of 330 µmol m^{-2} s^{-1}. Another burst of saturating light at Fs state was used to measure F'm. Once again reaching the steady state, leaves were exposed to far-red radiation light to measure F'o. Calculations of various parameters of chlorophyll fluorescence were carried out using calculations were carried out according to Schreiber et al. (1986).

Variable fluorescence (Fv)

Fv = (Fm-Fo)

The maximum quantum efficiency of PSII (Fv/Fm)

Fv/Fm = (Fm-Fo)/Fm

Photochemical quenching (qP)

(qP) = (Fm'-Fs)/(Fm'-Fo')

Non-photochemical quenching (qNP)

(qNP) = 1-(F'm-F'o)/ (Fm-Fo)

2.6.3 Extraction and separation of photosynthetic pigment

Pigment changes due to salt stress in rice plants were qualitatively and quantitatively determined using HPLC (High-Pressure Liquid Chromatography, Waters), according to Sharma and Hall (1996). The chemicals used were of HPLC grade (Merck specialist,

Lichrosolv India). Fresh leaf tissue of 200 mg was finely homogenized using acetone (100%) in a pre-cooled mortar and pestle. The final volume was made to 1.5 ml and incubated at 4°C overnight by adding a few Butyls Hydroxy Toulene (BHT) crystals. The homogenized was centrifuged (HERMLE Labortechnik GmbH, Germany) at 7000 rpm for 10 min at 4ºC and filtered through 0.2 µm nylon filter (Pall Pharmalabs, USA) to a new microcentrifuge tube and stored at -20ºC for further HPLC analysis. A sample volume of 20 µl was loaded for analysis in the HPLC system and separation was carried out using a C18 column (Water Spherisorb ODS 5 µm, 4.6 mm x 250 mm). The solvent system used for separation was a linear gradient of 0-100% ethyl acetate in acetonitrile/water (9:1) over 30 min with a flow rate of 1.2 ml/min at 25°C, and the peaks were detected using a Phase Diode Array (PDA) detector (Waters 2996) at 445 nm, and qualitative determination of each peak was done based on peak spectra, retention time, and available standards. β-carotene was used as an external standard to calculate the quantity of pigments on the peak area basis, and pigment content was expressed as µg g^{-1} FW.

2.7 Electrolyte leakage (EL)

It was determined with Electrical Conductivity (EC) meter. Fresh leaves tissue of 0.5 gm was washed three times with distilled water to remove surface contaminants and then placed individually in tubes containing 10 ml of Milli Q water. The vials were incubated at room temperature (25ºC) for 4 h. EC of the bathing solution was measured after incubation (E_1). The same tubes with leaf samples were then placed in a water bath at 100ºC for 30 min, and the second measurement of electric conductivity (E_2) was taken after cooling the solution to room temperature. The ion leakage was calculated according to Lutts et al. (1996).

$$\textbf{Electrolyte leakage (\%)} = (E_1 / E_2) \times 100$$

Where E_1 - Electrolyte conductivity before boiling

E_2 – Electrolyte conductivity after boiling

2.8 Determination of ROS generation

Excess salt induces oxidative stress by generating ROS in plants such as $O_2^{\cdot-}$, H_2O_2, and OH• production via the Haber Weiss-Fenton reaction and were measured spectrophotometrically.

2.8.1 Determination of hydrogen peroxide (H_2O_2)

Total H_2O_2 content was estimated according to Sagisaka (1976). Fresh leaf tissue of 200 mg was homogenized at 4°C and extracted in 5 ml of 5% trichloroacetic acid (TCA, Fischer Scientific, India). The Homogenate was centrifuged at 6000 rpm for 10 min at 4°C. The reaction mixture contained 40 µl of 50% TCA, 20 µl of 2.5 M potassium thiocyanate (KSCN, Himedia) and 40 µl of ferrous ammonium sulphate ($(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, Himedia) were added to the supernatant to determine the H_2O_2 level. The absorbance was recorded at 480 nm using a UV-Visible spectrophotometer. The concentration was determined using a calibration curve with a 10 mM H_2O_2 stock solution and expressed as µmol g^{-1} sample FW.

2.8.2 Determination of hydroxyl radical (OH•)

OH• radical assay was carried out as described by Aruoma et al. (1988) with modification von Tiedemann (1997). Briefly, Thiobarbituric Acid Reactive Substances (TBARS) formed by deoxyribose degradation are spectrophotometrically measured at 532 nm. Fresh tissue of 200 mg was finely ground in 1.5 ml of 0.05 M phosphate buffer with pH 7 and centrifuged at 4ºC for 10 min at 7000 rpm. 0.5 ml of supernatant was added to 1 ml of 25 mM phosphate buffer containing 2.5 mM 2-deoxyribose and was incubated for 1 h at 35°C in the dark. After the incubation, 1 ml of 1% thiobarbituric acid (TBA) and glacial acetic acid (GAA) (Merck) was added to the reaction mixture and incubated at 95ºC for 10 min in the dry bath (WiseTherm HB-48P), cooled immediately on ice, and absorbance was read at 532 nm using a UV-Visible spectrophotometer (UV2450, Shimadzu, Japan). Absorbance was recorded at 532 nm after cooling the reaction mixture. The concentration of OH• content was expressed as absorbance units per gram sample FW (absorbance ×1000).

2.9 Oxidative damage caused by ROS

2.9.1 Determination of lipid peroxidation (MDA)

A slightly modified protocol of Thiobarbituric Acid Reactive Species (TBARS) assay by Sankhalkar and Sharma (2002) was used to measure the leaf lipid peroxide. Extraction of fresh leaf tissues (500 mg) was carried out using 5 ml of 1% TCA (Fisher scientific India). The contents were centrifuged for 5 min at 5000 rpm (Elteck, TC 4100 D). To 1 ml of supernatant, 2.5 ml of incubation buffer containing 0.15 M NaCl, 0.05 M Tris HCl, and 1.5 ml of 0.8% (w/v) TBA in 20% TCA was added. The resulting mixture was

vortexed and then heated at 95ºC in a water bath for 30 min. Absorbance was recorded at 600 and 532 nm, after cooling, using a UV-Visual spectrophotometer (Shimadzu UV-2450). The nonspecific turbidity was corrected by subtracting absorbance at 600 nm, and the concentration of MDA was calculated by the extinction coefficient (155 mM^{-1} cm^{-1}), and the results were expressed as nmol g^{-1} FW.

2.9.2 Protein oxidation assay (Carbonyl content)

Oxidative stress induces protein oxidation in the form of carbonyls detected by the adduct formation between Dinitrophenyl Hydrazine (DNPH; Himedia, India) and carbonyl group (side chain aldehydes and ketones), which are measured spectrophotometrically. The carbonyl content was measured with 10 mM 2,4-dinitrophenylhydrazine in 3 M HCl and incubated at room temperature with regular vortexing for 1 h. The samples were again precipitated with 2 ml of 20% TCA and centrifuged at 7000 rpm for 10 min. The resultant pellet was washed with ethanol: ethyl acetate (1:1) to remove excess reagent. The final pellet was dissolved in 1ml of 6M guanidine –HCl, and carbonyl content was estimated at 370 nm (E= 0.022 μM^{-1} cm^{-1}) and carbonyl content calculated by the extinction coefficient according to Reznick and Packer (1994). Protein quantification was done according to the Bradford method (1976).

Protein carbonyl (nmol/ ml) = Calculated average (CA) / 0.022 $\mu M^{-1}cm^{-1}$ (a/b)

Where, Extinction coefficient = 0.022 $\mu M^{-1}cm^{-1}$

a= volume of sample used (µl)

b= volume of Guanidine HCl (µl)

2.10 Measurement of enzymatic and non-enzymatic antioxidants

Enzyme-based antioxidants like SOD, CAT, and APX support the organisms by keeping the ROS generation in check or regulating the detoxification process against oxidative stress and also serve as a damage repair mechanism. Briefly, the tissue homogenate in 50 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA-Na_2 and 2% w/v PVPP was used to determine enzyme activity. Protein quantification was done according to the Bradford method (1976). Leaf tissue (0.2 g) was homogenized in 2 ml of 0.5 M phosphate buffer (pH 7.8) and centrifuged for 10 min at 10,000 rpm at 4ºC. The resulted supernatant was used for the protein estimation. 200 µl sample was mixed with 1 ml Bradford's reagent, vortexed, and

incubated for 5 min at RT and absorbance was measured at 595 nm. The protein content were determined using bovine serum albumin (BSA).

2.10.1 Determination of Superoxide dismutase (SOD) antioxidant activity

The Superoxide dismutase activity (SOD) was measured by following the method described by Boveris (1984). Leaf tissue (0.2 g) homogenized in 1.5 ml of 50 mM sodium phosphate buffer (pH 7.8) centrifuged at 10,000 rpm for 1 min at 4^{o}C. The supernatant was used to determine the antioxidant activity. Reaction one consisted of 10 mM Na_2CO_3, 10 mM sodium phosphate buffer, 6 mM disodium EDTA, and 4.5 mM epinephrine. Reaction two consisted of sample extract instead of buffer. Reaction with water was considered blank using a UV visible spectrophotometer (UV 2450, Shimadzu, Singapore) at 480 nm. The protein concentration of enzyme extract was measured using the Bradford method at 595 nm, and the SOD activity was expressed as SOD activity (ΔA) min^{-1} mg^{-1} protein.

2.10.2 Determination of Ascorbate peroxidase (APX) Activity

The activity of ascorbate peroxidase (APX) was determined according to Nakano and Asada (1981). The reaction mixture contained 0.05 M Na–phosphate buffer (pH 7), 0.5 mM ascorbate, 0.1 mM EDTA, 1.2 mM H_2O_2, and 0.1 ml of tissue homogenate in a final assay volume of 1 ml. Ascorbate oxidation was followed at 290 nm. The concentration of oxidized ascorbate was calculated using the extinction coefficient ($\varepsilon = 2.8$ mM^{-1} cm^{-1}). One unit of APX was defined as 1mmol/ml ascorbate oxidized per minute. The protein concentration of enzyme extract was measured using the Bradford method at 595 nm.

2.10.3 Determination of Catalase (CAT) Activity

Catalase estimation was done,according to Beers and Sizer (1953). A fresh sample of 200 mg was homogenized using 2 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 0.5 mM EDTA and 2% PVP. Homogenate was carried out by centrifugation at 6000 rpm for 10 mins at 4°C. Assay volume of 3 ml containing 2 ml 100 mM K-phosphate buffer (pH 7.0), 900 µl 200 mM H_2O_2, and 100 µl enzyme. The decomposition of H_2O_2 was measured at 240 nm by observing a decrease in absorbance using a UV–vis spectrophotometer (UV–2450, Shimadzu, Singapore) with an extinction coefficient of 0.036 mM. The

protein concentration of enzyme extract was measured using the Bradford method at 595 nm, and enzyme activity was reported as µmol H_2O_2 oxidized $min^{-1}mg^{-1}$ protein.

2.10.4 Determination of Ascorbic acid (AsA)

AsA was determined according to the method of Kampfenkel et al. (1995). Fresh leaf tissue (0.1 g) was ground with 1 ml of extraction buffer (0.1 M HCl + 0.1 mM EDTA). The mixture was centrifuged at 12000 rpm for 2 min (Eppendorf, centrifuge 5804), and the supernatant was used for the ascorbate assay. The reaction mixture contained 100 µl of a sample, 100 µl of 0.4 M phosphate buffer (pH 7.4), 400 µl of color reagent (containing two solutions [A] TCA (4.6%), H_3PO_4 (15.3%), $FeCl_3$ (0.6%) and [B] 2, 2 dipyridyl (4%) in 70% ethanol, in the ratio of 2.75:1) and 50 µl of 0.5% N-ethylmalemide. The mixture was incubated at 42°C for 45 min, and absorbance was measured at 520 nm. Ascorbic acid (Hi-media) was used as a standard, and the results were expressed as µmol g^{-1} FW.

2.10.5 Determination of Proline (Pro) content

Determination of proline content was carried out according to Bates et al. (1973). Leaves (0.5 g) homogenized in 5 ml of 3% sulfosalicylic acid in mortar and pestle and centrifuged at 5000 rpm for 5 min. About 1ml of supernatant was taken in a test tube, and added with 1ml of acid ninhydrin with glacial acetic acid was added and mixed thoroughly by vortexing for 3min. The reaction mixture was incubated in a water bath at 95^{o}C for 1 h. After cooling, the mixture was added with 10 ml toluene and mixed vigorously and kept to settle it to settle for 30 min. Absorbance was read at 520 nm using a spectrophotometer against toluene as a blank. Proline concentration was calculated using the L-proline standard and expressed as µmol g^{-1} FW.

2.11 Fatty acid analysis using GC-MS

2.11.1 Lipid extraction and saponification

Total lipid extraction was carried out according Turnham and Northcote (1982). Plant leaf tissue (0.5 g) was boiled over a flame with isopropanol to removed excess chlorophyll, and tissue was ground using liquid nitrogen. Tissue extraction was carried out using chloroform and methanol (1:2 v/v). The supernatant was decanted, and the residue was washed again with 1:2 v/v chloroform and methanol, pooled and centrifuged the supernatant was centrifuged for 5 min at 5000 rpm. For saponification, 1 ml of

supernatant that was taken and the the reaction volume was made up to 15 ml by adding (4 ml dH_2O + 5 ml chloroform + 5 ml KCl solution) and separated using a separating funnel. The solvent phase was collected, dried with nitrogen, dissolved in 1ml of chloroform containing BHT, and stored at -20°C.

2.11.2 Acidification

Two conical flasks labeled A and B were taken and connected with a glass tube, and 30 ml of methanol was added to flask A and placed on a magnetic stirrer. To flask B, 10ml of concentrated sulphuric acid and 5 g of ammonium chloride were added and incubated for 15-20 min. Methanolic HCl of 5ml was taken from flask A and titrated against 1 M NaOH in the burette, and Normality (N2) was calculated. The sponified dried lipid sample was dissolved in 5 ml of MeOH-HCl, kept at 70-80°C in an oven with cap loosened for 10 min and further for 2 h with tightly closed, to this 5ml of dH_2O and 2 ml hexane were added, followed by cooling and vortexed for 90 sec, and solvent was allowed to separate; hexane was fractioned to another tube and same was repeated 3 times. Following the collection, 5 ml of sodium bicarbonate was added to the hexane and vortexed. The mixture was allowed to settle, and hexane was collected in a fresh tube; 5ml distilled water was added to the tube and vortexed for 15 sec, and hexane was sequentially collected in a fresh tube, and 2-3 crystals of fused $CaCl_2$ were added to it, and hexane was aliquoted from the tube without disturbing $CaCl_2$, dried under N_2 and stored at 4°C and dissolved in 100 µl hexane prior to GC-MS analysis.

2.11.3 Sample run specification and peak identification

FAME samples (1µl) profiling was carried out using gas chromatography-mass spectroscopy. The analysis was completed using a GC-MS system (Perkin Elmer Clarus 600) equipped with an Elite-5 MS Silica Capillary column (30 m*0.25 mm). The carrier gas helium (99.999%) was used at a 1ml/min flow rate. The injection port temperature was 250^oC, and the column temperature program was as follows; 50^oC for 2 min, followed by 270^oC for 5 min. The MS condition included an EI ion source temperature of 230^oC.

The separated components were tentatively identified by comparing their mass spectra with those in the NIST08 MS LIBRARY (National Institute of Standard and Technology, USA) and comparing their retention indices (RIs). The RIs were calculated relative to a C7-C30 alkane standard (Sigma Aldrich, St Louis, MO, USA) separated on the Elite-5

MS Silica Capillary column under the same GC-MS analysis condition. Each component was quantified based on the comparison of its peak area with that of the external standard, and the content is expressed as the mg g^{-1} FW.

2.12 Thylakoid membrane proteins (SDS - PAGE)

2.12.1 Isolation of chloroplast

Leaves from rice plants were used to isolate chloroplasts, according to Sharma & Singhal (1992). Leaves (4 g) were finely chopped into fine pieces and homogenized in (Grinding media) containing sorbitol tricine (0.33M), $MgCl_2$ (1mM), $MnCl_2$ (1mM), NaCl (1mM), and EDTA (1mM) (pH 7.2) using a homogenizer (Miccra-D-9). Homogenate was thoroughly filtered and centrifuged at 5000 rpm for 10 min. Pellet was washed twice with (resuspending media) containing sorbitol, tricine, $MgCl_2$, $MnCl_2$, NaCl, and EDTA (pH 7.8) and then resuspended in the same media. All the experimental steps were performed at 4°C.

2.12.2 Preparation of protein sample for SDS – PAGE

Chloroplasts isolated were resuspended in 2X sample buffer (Himedia) cantaining 4% SDS, 20% glycerol, 0.004% bromophenol blue, 0.12M Tris-Cl (pH 6.8), and 10% β-mercaptoethanol and incubated overnight at 4°C for complete solubilization of protein samples. This sample was heated at 90°C for 3 min and cooled immediately by transferring the sample onto the ice. Samples were centrifuged at 10,000 rpm for 1 min and the supernatant equivalent of 12 µg protein was used as a source of thylakoid protein. Protein concentration was measured using the Bradford method at an absorbance of 595 nm.

2.12.3 SDS PAGE and its analysis

Protein sample 12 µg equivalent was electrophoresed on 5% stacking and 15% resolving SDS polyacrylamide gels (Table 2.12.3) and developed initially at 50 V for 30 min and subsequently at 120 V for 2 h with a PROTEAN II X1 2-D Cell (Bio-Rad). Gels were subsequently stained with a staining solution containing 0.1% Coomassie brilliant blue R 250 (CBB), 10% Glacial Acetic Acid (GAA), and 50% methanol for overnight. Excess stain was destained using destainer solution I containing 10% GAA and 40% methanol for 2-3 h and transferred to destainer solution II containing 7% GAA and 5%

methanol. Finally, the gel was placed in distilled water. Gels were scanned using Bio-Rad Gel Densitometer (GS -800) and analyzed using Quantity One software from Bio-Rad.

Table: 2.12.3: Composition of SDS-PAGE gel.

Sr. No.	Composition	10 ml of resolving gel	5 ml of stacking gel
1	Acrylamide/Bisacrylamide (30%/0.8% w/v; Sigma, USA)	4ml	0.67ml
2	1.5M Tris (pH 8.8); Himedia, India	2.6ml	-
3	0.5M Tris HCl (pH 8); Himedia, India	-	1.25ml
4	10% (w/v) (SDS, Sigma, USA)	0.1 ml	0.05 ml
5	10% w/v (APS, Sigma, USA)	0.1ml	0.05ml
6	TEMED	0.01ml	0.005ml
7	Double distilled water	3.2ml	2.97ml

2.13 Proteomic study (Swath Analysis)

Proteomic studies on rice leaves were carried out to determine and establish the effects of increasing salt stress on the differential expression pattern of proteins according to Korwar et al. (2015).

2.13.1 Protein extraction and quantification

Fresh leaf samples (200 mg) were ground using liquid nitrogen, and whole protein was extracted in 1.5 ml of 10% TCA in 100% acetone and incubated at -20°C for 1 h. The reaction mixture was incubated for 15 min at RT on slow vortex and centrifuged at 12000 rpm for 15 min at 4°C. The pellet was resuspended in 1.5 ml of 80% methanol with 0.1% ammonium acetate and incubated for 15 min at RT on slow vortex and centrifuged at 12000 rpm for 15 min at 4°C. Extraction buffer (80% acetone containing 2% β Mercaptoethanol)was added to the pellet and vortexed for 30 sec and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was collected in to a new tube, and 1ml of Tris saturated phenol, pH 8.0 (Sigma, USA) was added and vortexed for 1 h and centrifuged at 5000 rpm for 15 min at 4°C. The phenol layer (upper transparent layer) was collected in a fresh tube and 12 ml of 0.1 M ammonium acetate (Sigma, USA) in 80% methanol (LC-MS Chromasolv®, Sigma, USA) with 2% β-ME was added and

incubated at -20°C for overnight. The reaction mixture was centrifuged at 5000 rpm for 20 min at 4°C. The protein pellet was suspended and solubilized in 100% methanol containing 0.1% ammonium acetate by gentle pipetting and transferred to a protein LO-BINDING tube (Eppendorf, Germany) and centrifuged at 4000 rpm for 15 min at 4°C. The step was repeated twice. After methanol washing, the pellet was resuspended in 80% acetone (LC-MS Chromasolv®, Sigma, USA) with 0.2% β-ME and vortexed for 15 sec and centrifuged at 10,000 rpm for 10 min at 4°C to removed all the acetone traces. The supernatant was discarded, and the pellet was drained and air-dried at RT in fume food for 1 h. Pellet was resuspended in 50 µl of Rapigest (Waters, USA), vortexed for 15 sec, and incubated for 1 h at 60°C under agitation Thermo mixer (Eppendorf, Germany) for complete solubilization and centrifuged at 4000 rpm for 15 min at 4°C. The supernatant was recovered in fresh LO-BINDING tubes and used for protein quantification using Bradford reagent (BioRad).

2.13.2 Trypsin digestion

200 µg of protein extracted in Rapigest was heated at 80°C for 20 minutes and reduced with 3 µl DTT (1mM stock in 50 mM ammonium bicarbonate) at 65°C for 15 minutes. This was alkylated with 3 µl IAA (2 mM stock in 50 mM ammonium bicarbonate) at room temperature in the dark for 30 min and subjected to proteolytic digestion by 2 µg trypsin (0.2 µg/µl stock in 50 mM ammonium bicarbonate) at 37°C with gentle shaking for 16-18 h. The digested peptides were desalted using Pierce C-18 Spin Columns (Thermo Scientific, USA) as per the manufacturer's protocol.

2.13.3 Elution of protein

Each digest was injected onto a C18 reverse-phase column (dimensions: 100×0.3mm, 3µm, 120 Å) of a micro-LCD 200 liquid chromatography system (Eksigent Technologies, USA) coupled to a Triple TOF 5600 mass spectrometer (SCIEX, USA). Peptides were separated over a 95-minute gradient of 3-40% acetonitrile in water with 0.1% formic acid at a flow rate of 7 µl/min. The spectral library was first generated for label-free quantification using SWATH by acquiring all samples in Information Dependent Acquisition (IDA) mode over a mass peptide range of 400-1250 m/z. Accumulation time for MS was 250 ms, while that for MS/MS was 70 ms. Peptide fragmentation was performed using rolling collision energy, and MS/MS was conducted over a mass range of 100-2000 m/z. Three replicate SWATH runs were acquired for each sample. The

sample was added with β-galactosidase standard digest as an internal standard in all the runs. Briefly, 1 pM standard stock was prepared in MS-grade water. This internal standard was added to the sample digest to obtain a final load of 500 fM for each IDA run and 200 fM for each SWATH run. For each sample, 3.5 µg and 2 µg digests per 5 µl injection were loaded for IDA and SWATH runs, respectively.

2.13.4 Sample run specification

The IDA and SWATH analysis was performed for the control and salt-treated leaf tissues of both rice varieties. The IDA data were searched against the UniProt *Oryza Sativa* Indica database using ProteinPilot^{TM} version 5.0 software. The result generated was used in PeakView v2.2 software as a spectral library. The SWATH runs were processed using 50 ppm error, 4 min retention time window, 99% confidence, and 1% False Discovery Rate (FDR). For quantitative and statistical analysis, the processed data containing the peak areas for all proteins, peptides, and ions were further exported to MarkerViewTMv1.2.1. The data across the runs were normalized using the total area sum, and a t-test was performed for statistical evaluation. Only the proteins with a p-value <0.05 were considered, and those showing more than >1.3 fold change difference of were reported as differentially expressed.

2.14 Transcriptomic study

Genes are responsible for synthesizing the functional gene product. Determining the changes in their expression level will provide information about the molecular responses induced by the NaCl stress in rice plants. These changes can further relate to the biochemical parameters to determine the response to increasing NaCl. For this analysis, RT PCR was used, and the purity of the product was determined using the Tm curve of the product.

2.14.1 RNA Extraction

Fresh 100 mg of leaf tissue was ground in liquid nitrogen, and RNA extraction was carried out with 500 µl TRIzol (Ambion ≠15596018). Homogenate was mixed by gentle inversion and incubated for 5 mins at RT, and added 100 µl chloroform ($CHCl_3$, Merk Emplura). The contents were vortex vigorously and incubated at room temperature for 3 min, then centrifuged at 5000 rpm for 15 min at 4°C (Hermle, Z32HK). The upper aqueous phase was transferred to a new sterile tube, to which 250 µl isopropanol

(Himedia, India) was added and gently mixed for 25-30 sec and allowed to stand for 30 min at -20^{o}C. The samples were centrifuged for 10 min at 5000 rpm at 4°C, and the supernatant was discarded. RNA pellet is gently washed with 70% ethanol and centrifuged pellet. The supernatant is carefully discarded, and the pellet is dried inside the hood at 37°C. The RNA pellet was resuspended with ultrapure nuclease-free molecular-grade water, and RNA was quantified using the nanodrop with ultrapure water as a blank.

2.14.2 cDNA preparation

According to the manufacturer's instructions, the cDNA was synthesized with 5 µg of RNA using the cDNA isolation kit (Thermo Fischer Scientific, applied Biosynthesis, 4368814) with an oligo dT primer. The total reaction volume was set at 25 µl, and cDNA synthesis was performed in four-stage; the first stage at 25^{o}C for 10 min fallowed by the second stage at 37^{o}C for 60 min, the third stage at 85^{o}C for 5 min, and the last stage at 5^{o}C for 5 min. The prepared cDNA samples were used for the gene expression study.

2.14.3 Real-Time PCR

The expression profile of antioxidant enzymes (SOD and APX), Na^{+} transporter gene (OsHKTI;1), K^{+}/Na^{+} co-transporter (OsHKT2;4), and proline synthesis gene (OsP5CS1) were quantified using Bio-Rad, MJ Miniopticon Real-time PCR Detection System using (SYBR Green PCR master mix Cat no.:4367659). According to the manufacturer's instructions. The total reaction volume of 10 µl contained 2.5 µl cDNA, 0.5µl primer, 5 µl 2X master mix, and 2 µl of ultra-pure water.

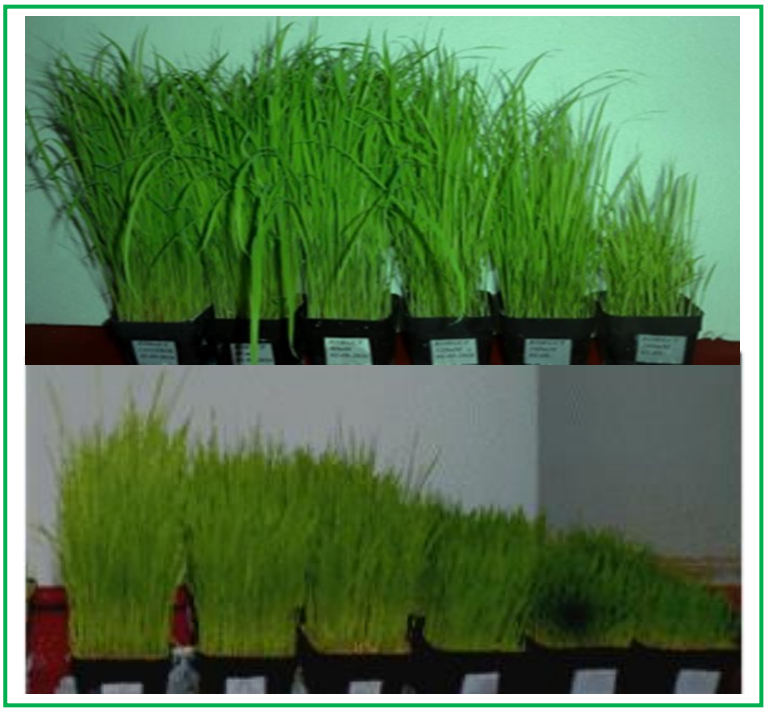
Table 2.14.1: List of primers (Reverse and Forward) used to study gene expression by real-time analysis. SOD = Cu/Zn Superoxide dismutase, APX = Ascorbate peroxidase of thylakoid, OsHKT2;4 = K^+/Na^+ co-transporter, OsHKTI;1=Na^+ transporter gene and OsP5CS1 = Proline synthesis gene.

Sr. No.	Primer name	Sequence
1	SOD	Cu/Zn SOD -1-F GGTGTCCTGCAGCAGATAGAG Cu/Zn SOD -1-R CAGCCAGACCCCAAAAGTGA
2	APX	APX- 1-F CAGCCAGACCCCAAAAGTGA APX - 1-R TCCGTGAAGTAAGAGTTGTC
3	OsHKT2;4	K^+ transporter -1-F AGATGTTCCTTTCCTTTCTTGGC K^+ transporter -1-R AGATGTTCCTTTCCTTTCTTGGC
4	OsHKT1;1	Na^+ transporter -1-F CCTTTTGCATCTTCACAGCA Na^+ transporter -1-R ATACGCATAGCCGCAAGAGT
5	OsP5CS1	Proline synthesis-1 F-ATTGGGTGCTGAGGTTGGCATAA Proline synthesis- 1-RCGACATCCTTGTCACCATTCACCA

2.15 Statistical analysis

Statistical analysis for each treatment was conducted with three replicates, and the results were presented as mean ± SD (standard deviation). The means of response obtained were statistically analyzed by one-way ANOVA using Graph Pad Prism (Version 5.0). A statistically significant difference is reported; the *P*-value is ≤ 0.05. The correlation analysis between different parameters was further confirmed using STATISTICA (data analysis software, Version 8.0), with a significant value calculated. Graphs were prepared using the Microsoft Excel program. Principal component analysis (PCA) was performed using XLSTAT software. The first two principal components were used to derive PCA-biplot, and the possible associations among the genotypes and measured physiological and biochemical traits were determined.

CHAPTER 3



RESULTS

“Success is the result of perfection, hard work, learning from failure, loyalty, and persistence.”

Colin Powell

In the present chapter, two high-yielding rice varieties, 'Korgut' (salt-tolerant) and 'Jaya' (salt-sensitive), were assessed with respect to their response to salinity stress (40-200 mmol/l NaCl) at the early vegetative stage. In response to increasing NaCl treatment with regards to the assessment in the growth, morphology (SEM and TEM), photosynthesis, accumulation of Na^+, K^+, Cl^-, and Na^+/K^+ ratio. To further understand the tolerance mechanism, we have also attempted Na^+ and K^+ transporter gene expression studies. We have also focused on ROS generation subsequent to different oxidative stress and both activity and expression level of antioxidant enzymes (SOD, APX, and CAT). In addition, we have also attempted proteomics (Swath) and lipidomic (GC-MS) analysis of both rice varieties under NaCl treatment. The main aims of our study were to have a better understanding and elucidate the fundamental mechanism involved in salt tolerance by comparing salt-sensitive and tolerant rice varieties and to investigate the relationships between physiological, biochemical, and molecular characteristics that may help select the traits to develop rice cultivars suitable to grow in coastal soil.

3.1 Effect of salt stress on plant growth and biomass

Root and shoot growth was measured after 21 days of the salinity treatment. As the salinity level increased from 40 to 200 mmol/l, there was a significant decrease in shoot lengths (32-70%) and root (29-78%) in the salt-sensitive 'Jaya' variety. 'Korgut' variety showed a relatively lesser decrease in the lengths of the shoot (15-54%) and root (16-55%) as compared to their control (Fig. 3.1, Table 3.1).

Shoot fresh and dry weight decreased significantly in both varieties as NaCl levels increased. 'Jaya' variety showed a decrease in shoot fresh weight by 59% and dry weight by 41% at 80 mmol/l NaCl concentration, while the 'Korgut' variety showed only 10% and 12% reduction in the shoot fresh and dry weight, respectively, at the same concentration of NaCl. However, the fresh and dry shoot weight in 'Jaya' was reduced to 69 and 68% and 48 and 32% in 'Korgut' when treated with 200 mmol/l NaCl concentration compared to their respective controls (Table 3.1). Similarly, root fresh and dry weight also significantly decreased in both varieties. The salt stress level of 80 mmol/l NaCl concentration reduced the root fresh and dry weight in the 'Jaya' variety to 26 and 42%, respectively, whereas in 'Korgut,' it reduced only 10 and 9% compared to the control (Table 3.1). At the highest salt stress level of 200 mmol/l, approximately 67% reduction in fresh and dry weights of root in the 'Jaya' variety was observed compared to

a decrease observed in fresh weight (45%) and dry weight (32%) in the root of salt-tolerant 'Korgut' variety (Table 3.1).

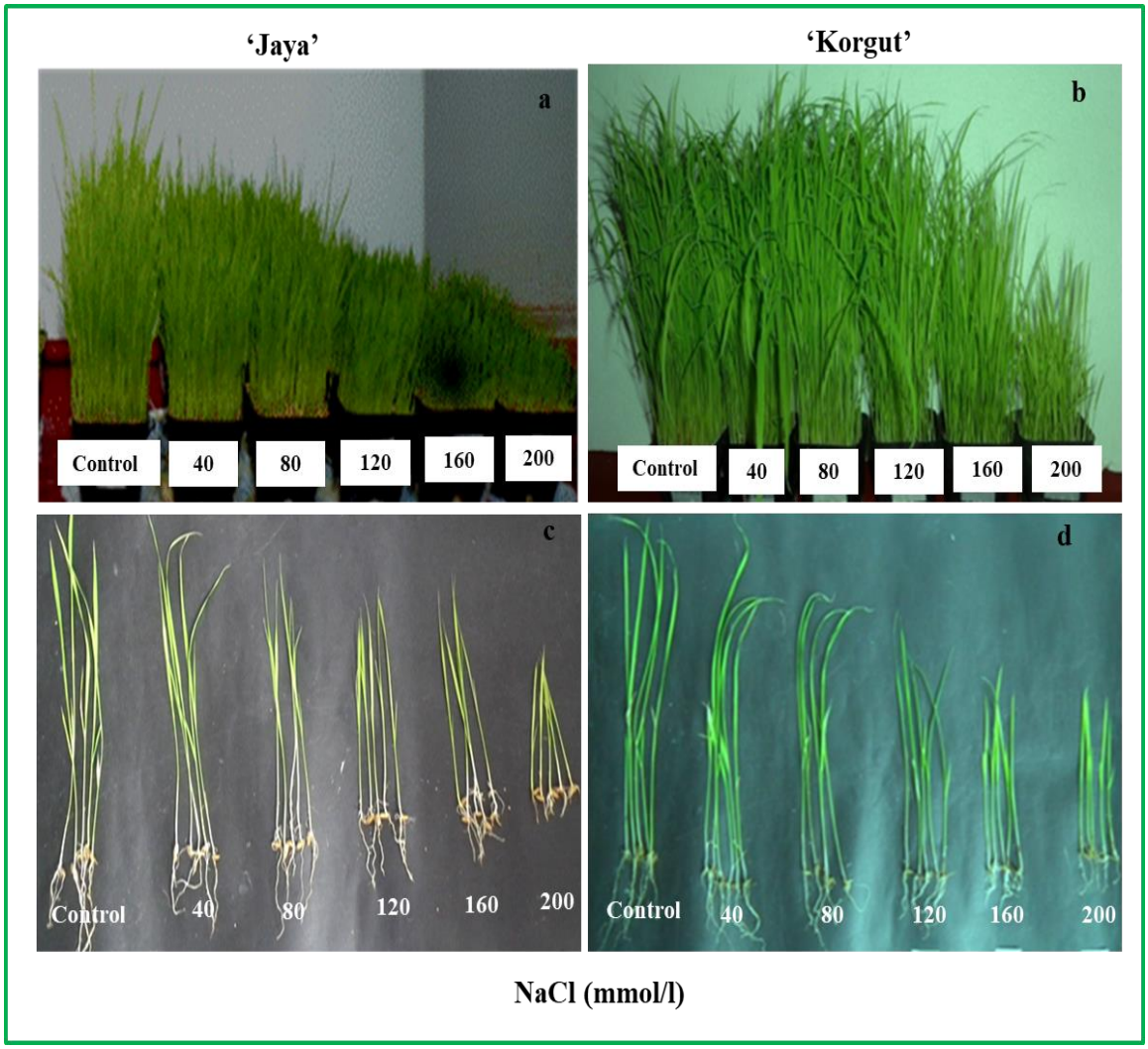


Fig. 3.1: Photographic image of 21 days old *Oryza sativa* 'Jaya' and 'Korgut' varieties grown in Hoagland solution supplemented with NaCl solution of 40, 80, 120, 160, and 200 mmol/l concentration showing growth (a-b) and; shoot and root length (c-d).

Table 3.1: Effect of Salt Stress (NaCl 40-200 mmol/l) on the shoot and root length and fresh and dry weight in 21-day-old 'Jaya' (salt-sensitive) and 'Korgut' (salt-tolerant) varieties of rice.

Rice Variety	Salt Stress (mmol/ l)	Growth (mm)			Biomass (g)			
		Shoot Length	Root Length	Root/shoot ratio	Shoot		Root	
					Fresh Weight	Dry Weight	Fresh Weight	Dry Weight
'Jaya'	0#	24±0.6	5±0.5	0.21	0.7±0.06	0.09±0.014	0.15±0.03	0.03±0.002
	40	22±0.2*	4±0.1**	0.18	0.4±0.02***	0.06±0.002**	0.13±0.02^{ns}	0.02±0.001***
	80	17±0.5*	4±0.1***	0.15	0.3±0.01***	0.05±0.004***	0.11±0.02^{ns}	0.01±0.001***
	120	14±0.6*	3±0.1***	0.15	0.3±0.02***	0.05±0.004***	0.09±0.01*	0.01±0.001***
	160	11±0.3*	2±0.2***	0.15	0.3±0.03***	0.04±0.005***	0.06±0.01***	0.01±0.001***
	200	7±0.7*	1±0.2***	0.13	0.2±0.0***	0.03±0.002***	0.05±0.01***	0.01±0.001***
'Korgut'	0#	40.6±2.2	7.71±0.42	0.17	0.92±0.0	0.18±0.01	0.21±0.02	0.02±0.001
	40	34.4±1.53**	6.47±0.3**	0.17	0.86±0.05^{ns}	0.16±0.02^{ns}	0.2±0.03^{ns}	0.02±0.001^{ns}
	80	31.27±1.7***	6.3±0.17***	0.17	0.73±0.04***	0.16±0.02^{ns}	0.19±0.03^{ns}	0.02±0.0^{ns}
	120	29.2±0.52***	5.56±0.4***	0.17	0.73±0.02***	0.14±0.03^{ns}	0.17±0.03^{ns}	0.02±0.0^{ns}
	160	24.73±1.1***	4.23±0.0***	0.16	0.65±0.0***	0.13±0.01^{ns}	0.12±0.02^{ns}	0.01±0.0***
	200	18.63±2.54***	3.43±0.25***	0.16	0.48±0.05***	0.12±0.02^{ns}	0.11±0.01^{ns}	0.01±0.001***

(*), (**), (***) indicates the significant at $p< 0.05$, $p< 0.005$, $p< 0.01$ respectively; ns – not significant. 0# (Control, i.e., without NaCl treatment). Standard deviation (±SD) indicates the means of three replicates.

3.2 Effect of salt stress on Relative water content (RWC)

The RWC was higher throughout the concentration of salt treatment in 'Korgut' than in 'Jaya'. We observed that RWC was decreased more in the 'Jaya' than in the 'Korgut' variety with increasing NaCl concentration. A linear decrease of 49, 62, and 73% in RWC was seen at 40, 120, and 200 mmol/l of NaCl treatment, respectively, in a 'Jaya' variety, compared to its control (Fig. 3.2). While in 'Korgut,' a decrease of only 4, 9, and 27% at the same concentration was observed compared to their control. However, RWC content was 2.8-fold higher in 'Korgut' than observed in 'Jaya' at the highest concentration (200 mmol/l of NaCl) (Fig. 3.2).

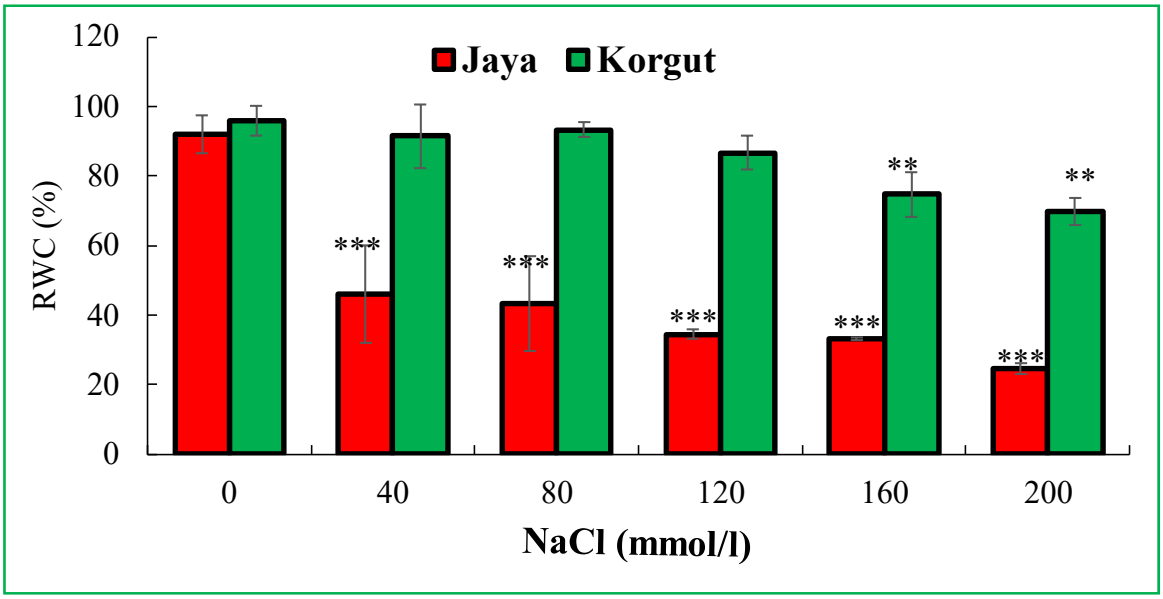


Fig. 3.2: Effect of NaCl on relative water content (RWC) in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (**), (***) indicates the significant at $p< 0.005$, $p< 0.01$ respectively. Standard deviation (±SD) indicates the means of three replicates.

3.3 Effect of salt stress on leaf morphology

3.3.1 External leaf morphology (SEM)

The effect of NaCl on the external morphology of leaf studied using a scanning electron microscope (SEM) showed a decrease in the number of trichomes (Fig. 3.3.1A; a-c) and an increase in the size of stomata in the 'Jaya' variety (Fig. 3.3.1A; d-f). Whereas morphological studies with the 'Korgut' variety showed an increase in the number and size of trichomes (Fig. 3.3.1B; a-f) but a decrease in the size of stomata (Fig. 3.3.1B; g-i) with increasing concentration of NaCl. SEM images also revealed a decrease in the abundance of the cuticular papillae on the adaxial leaf surface of the 'Jaya,' whereas there

was an increase in the cuticular papillae (cp) in the 'Korgut' variety at 120 and 200 mmol/l NaCl as compared to their respective controls.

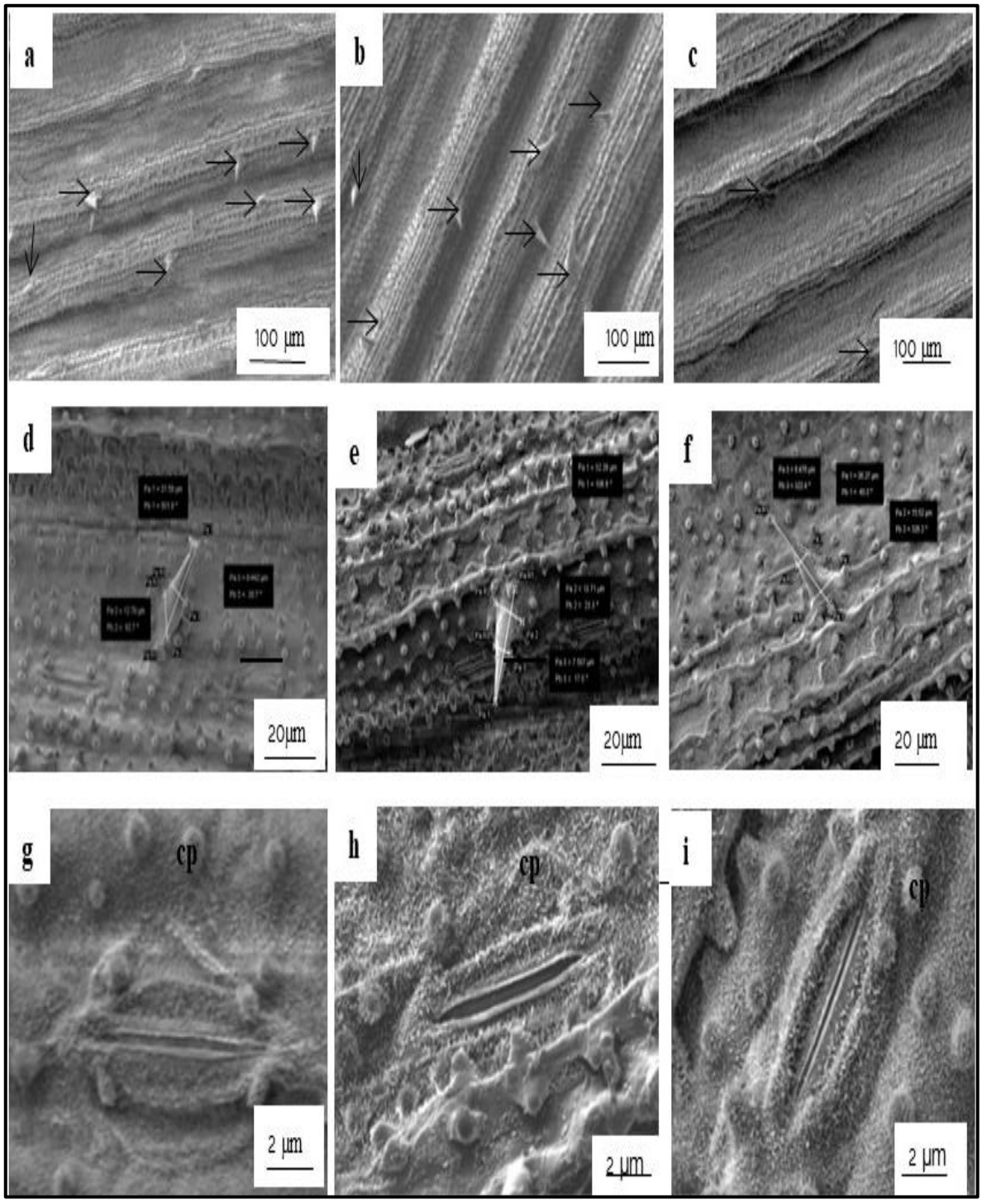


Fig. 3.3.1A: SEM images of adaxial leaf surface of 'Jaya' treated with NaCl for 21d, showing number (a-c) and size of trichome (d-f), size of stomata (g-i) in control (a,d,g), 120 (b,e,h) and 200 mmol/l NaCl (c,f,j). Cuticle papillae (cp) can also be seen in the images (e,f,g,h, and i).

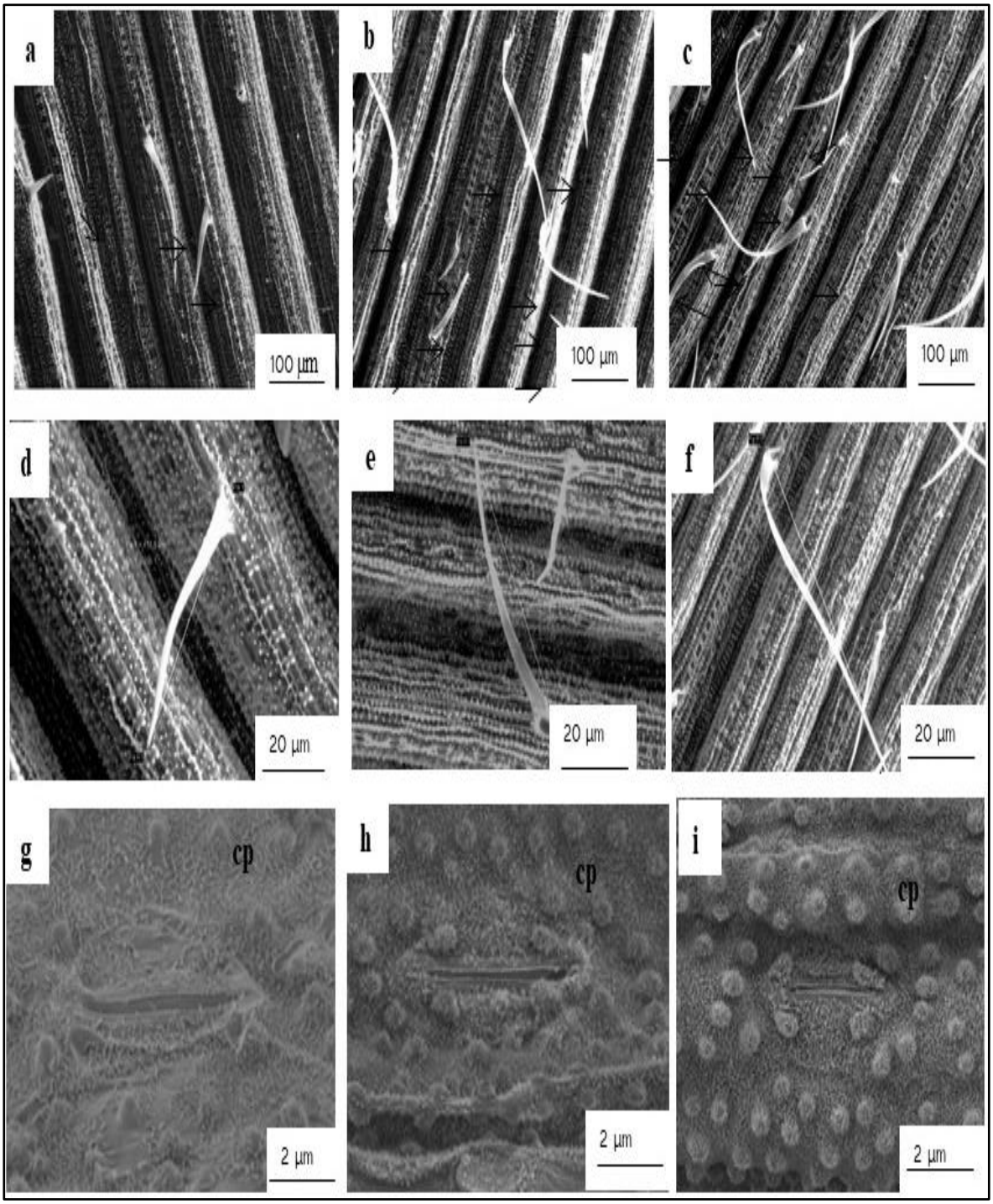


Fig. 3.3.1B: SEM images of adaxial leaf surface of 'Korgut' treated with NaCl for 21d, showing a number of trichomes (a-c), size of trichome (d-f) and size of stomata (g-i) in control (a,d,g), 120 (b,e,h) and 200 mmol/l NaCl (c,f,j). Cuticle papillae (cp) can also be seen in the images (g,h, i).

3.3.2 Internal leaf morphology (TEM)

The internal leaf morphology was observed in both varieties using TEM. Chloroplasts had a well-developed system of thylakoids and contained one or two small granules of starch in the 'Jaya' variety (Fig. 3.3.2A; a), while in 'Korgut' control, the chloroplast structure is far bigger (Fig. 3.3.2B; a). Salt treatment resulted in the clumping of chloroplast at the junction of the cell in 'Jaya' (Fig. 3.3.2A; b,c). Whereas in 'Korgut,' they are well spread out (Fig. 3.3.2B; b,c). Ultrastructure of chloroplast did not show any distortion of stacking of grana and no presence of myelin structure as the result of salt stress in the 'Korgut' (Fig. 3.3.2B; d,e,f), while 'Jaya' showed distortion and reduction in a number of the thylakoid membrane and grana with the presence of myelin like structure in the vacuole at higher concentration of salt. (Fig. 3.3.2A; d,e,f).

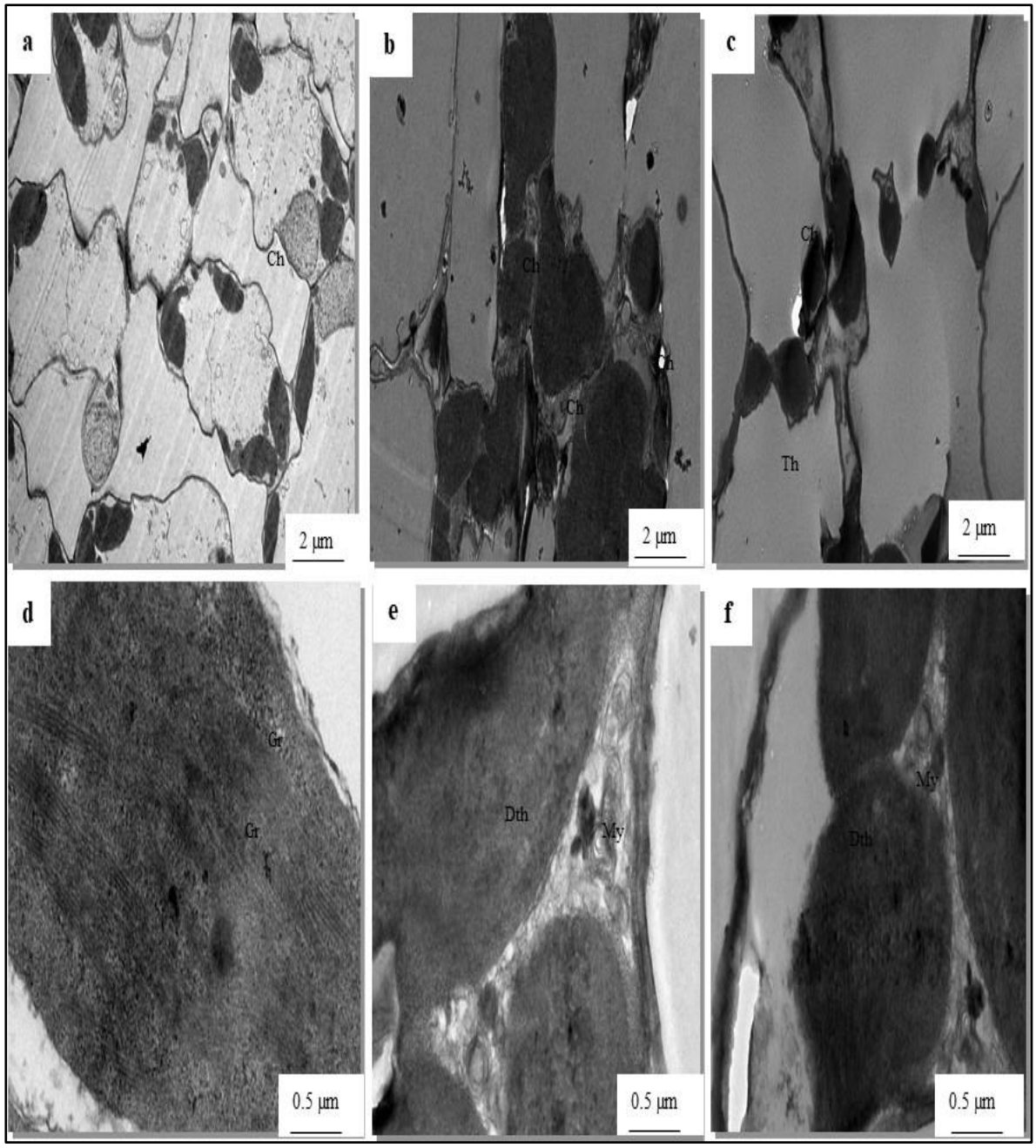


Fig. 3.3.2A: TEM images of 'Jaya' leaf treated with NaCl for 21d, showing whole mesophyll cell (a-c), and changes in thylakoid stacking (d-f) in control (a, d), 120 (b,e) and 200 (c,f) mmol/l NaCl. Thylakoid stacking (Th), grana (Gr), distortion of thylakoid membrane (Dth), and myelin (My). Myelin can also be seen in the images (e,f).

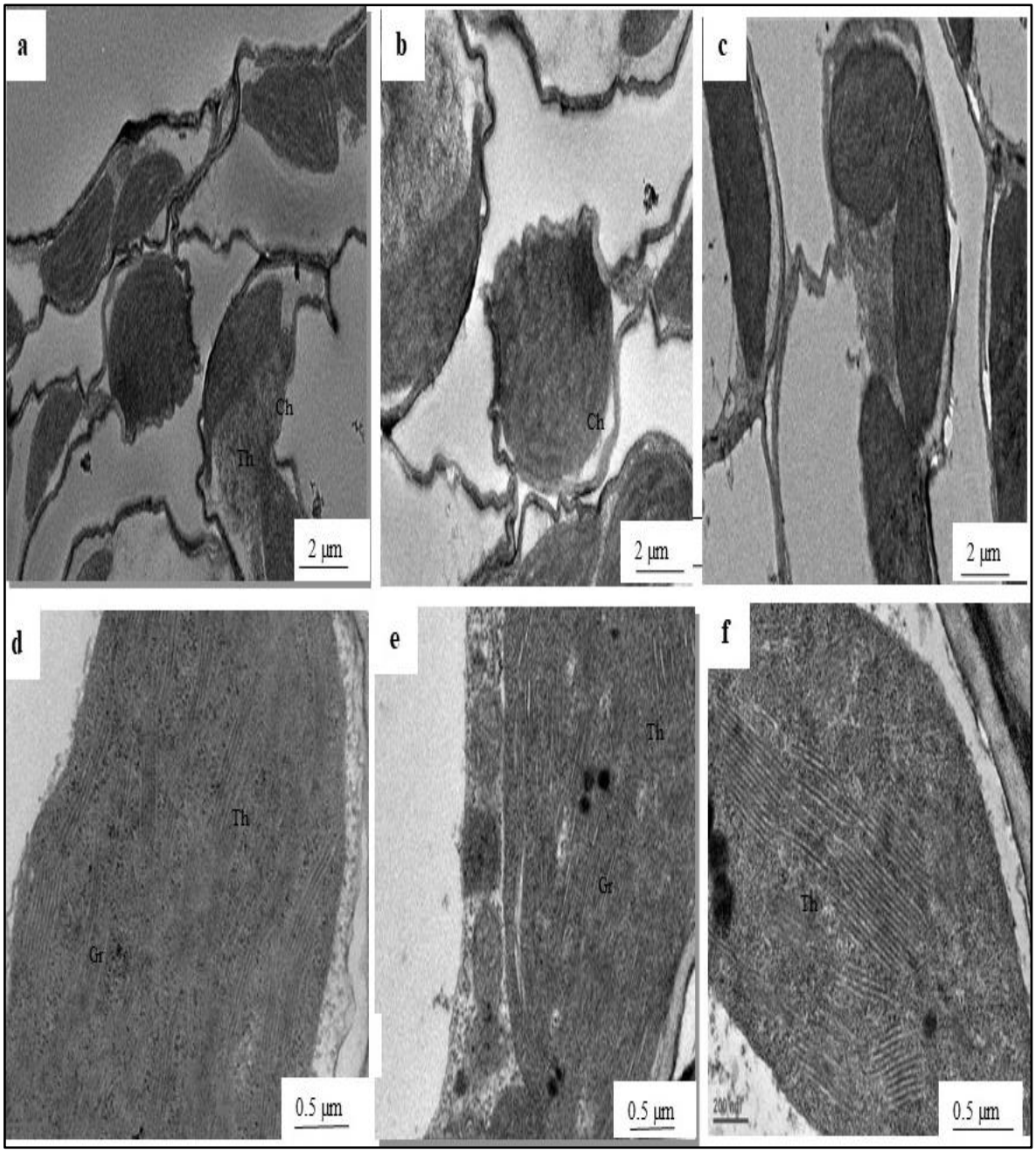


Fig. 3.3.2B: TEM images of 'Korgut' leaf treated with NaCl for 21d, showing whole mesophyll cell (a-c), and changes in thylakoid stacking (d-f) in control (a, d), 120 (b,e) and 200 (c,f) mmol/l NaCl. Thylakoid stacking (Th), grana (Gr), distortion of thylakoid membrane (Dth).

3.4 Effect of salt stress on element analysis

3.4.1 Sodium measurement (Na^+)

Salinity stress-induced excessive accumulation of Na^+ in leaves and roots of the 'Jaya' compared to 'Korgut' (Table 3.4.1). Higher Na^+ accumulation was seen in roots than in

leaves of both the varieties studied. Na^+ content in 'Jaya' leaves and root linearly increased to 16.88 and 20.27-fold, respectively, at the highest salt stress level (200 mmol/l) (Table 3.4.1). Similarly, leaves of the 'Korgut' variety showed a 15.26-fold increase in Na^+ accumulation compared to 25.51-fold observed in roots compared to control, indicating a relatively 1.67-fold higher accumulation of Na^+ in its roots compared to leaves. In comparison to 'Jaya,' Na^+ content in 'Korgut' leaves and root was 60% lesser at 200 mmol/l NaCl concentration (Table 3.4.1).

3.4.2 Potassium measurement (K^+)

K^+ content, on the other hand, decreased linearly in the root and leaves of salt treated 'Jaya' variety, whereas the K^+ content increased linearly with increasing salt stress in 'Korgut' compared to its control (Table 3.4.1). At the highest salt stress of 200 mmol/l, leaves and roots of 'Jaya' showed 57% and 44% reduction, respectively, in K^+ accumulation in comparison to its control, whereas the 'Korgut' showed a linear increase in K^+ to the extent of 11.2% in leaves and 931% in roots at the highest level of salt stress in comparison to its control (Table 3.4.1).

Approximately 97% reduction in the K^+/Na^+ ratio was observed in 'Jaya' grown at 200 mmol/l salt stress compared to the control. However, in 'Korgut,' the decrease in the K^+/Na^+ ratio was observed to be 93% in leaves and 82% in roots in comparison to its control. It was also observed the decreased K^+/Na^+ ratio was much greater at low concentrations in 'Jaya,' which was not observed in the case of 'Korgut' for the same concentrations of salt (Table 3.4.1).

3.4.3 Chloride measurement (Cl^-)

Salinity stress also caused a significant increase in Cl^- concentration in 'Jaya' than 'Korgut' (Table 3.4.1). Higher Cl^- accumulation was seen in roots than in leaves of both the varieties studied. Cl^- content in 'Jaya' leaves and root linearly increased to 3 and 5-fold, respectively, at the highest salt stress level (200 mmol/l). Whereas in the 'Korgut' variety, Cl^- content increased to 1.9-fold in leaves and 2.9-fold in root as compared to their control for the same treatment, showing a 57 and 52 % decrease in Cl^- content in leaves and root of 'Korgut' respectively, as compared to leaves and root of 'Jaya' at 200 mmol/l NaCl concentrations (Table 3.4.1).

Table 3.4.1: Effect of NaCl on sodium (Na^+), potassium (K^+), K^+/Na^+ ratio of leaves and roots in 'Jaya' and 'Korgut' varieties treated for 21d.

Rice Variety	Salt Stress (mmol l^{-1})	Na^+ (%)		K^+ (%)		K^+ / Na^+ Ratio		Cl^- (%)	
		Leaves	Root	Leaves	Root	Leaves	Root	Leaves	Root
'Jaya'	0#	100.00 ± 0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	7.49	1.89	100.00 ± 0.00	100.00± 0.00
	40	572.00 ±49.00*	476.00±13.82***	66.36±2.64***	92.60 ± 2.7^{ns}	0.86	0.39	104.00 ± 3.6^{ns}	130.00 ± 2.42^{ns}
	80	742.00±114.00**	672.00±49.11***	58.83±2.97***	75.89 ± 2.68^{ns}	0.60	0.24	137.00± 6.1^{ns}	138.00 ± 8.81^{ns}
	120	979.00±185.00***	786.00±106.13 ***	58.37±1.41***	60.51 ± 1.15 ***	0.45	0.23	187.00 ± 4.5***	179.00 ± 14.73^{ns}
	160	1500.00±184.00***	1475.00±65.65***	48.05±1.14***	45.62 ±7.34***	0.25	0.07	275.00 ± 6.24***	302.00 ± 67**
	200	1527.00 ±152.00***	2556.00±103.92***	43.22±1.07***	40.39 ±0.03***	0.22	0.04	334.00 ± 21***	488.00± 89.18***
'Korgut'	0#	100.00 ± 0.00	100.00 ±0.00	100.00±0.00	100±0.00	12.74	3.00	100± 0.00	100.00± 0.00
	40	257.00 ± 92.00*	406.00 ±11.46 **	105.00±2.00^{ns}	343.00±16.39***	5.62	1.05	116.34± 4.18^{ns}	127.78± 16.15^{ns}
	80	335.00 ±15.00**	566.00 ±34.49**	108.00±1.70**	352.00±4.62***	4.04	0.97	137.5± 16.68^{ns}	128.86± 31.48^{ns}
	120	403.00 ± 11.00***	677.00 ±5.2**	109.00±4.00***	663.00±31.83***	3.44	0.87	145.75± 26.69^{ns}	185.83± 46.79**
	160	855.00 ± 81.00***	1239.00 ±30.79***	117.00±4.00***	734.00±36.35***	1.64	0.61	181.12± 10.83 **	236.95± 53.9***
	200	1027.00 ±37.00***	1858.00±443.01***	119.00±5.00***	1032.00±77.43***	0.85	0.53	243.85± 30.06**	308.72± 28.76***

(*), (**), (***) indicates that the mean difference between salt stress response compared to control is significant $p< 0.05$, $p< 0.01$, and $p< 0.005$, respectively.0# (Control, without NaCl treatment). Standard derivation (±SD) indicates the means of three replicates.

3.5 Effect of salt stress on Photosynthesis

3.5.1 CO_2 fixsation

A significant decrease was observed in net photosynthetic rate (P_N), transpiration rate (*E*), and stomatal conductance (*gs*) at 200 mmol/l salt stress in both varieties (Fig. 3.5.1). The decrease in 'Jaya' was found to be linear with an increase in the salt concentration; however, in 'Korgut,' it showed no significant decrease till 80 mmol/l NaCl level and very little (7.5%) at 120 mmol/l NaCl. A comparatively higher reduction in the photosynthetic rate at 200 mmol/l NaCl was observed in salt-sensitive 'Jaya' (81%) than in salt-tolerant 'Korgut' (49.9%) (Fig. 3.5.1 a).

The transpiration rate also showed a similar trend. A linear decrease in transpiration rate of 82.28%, at the highest salt stress, was observed in 'Jaya' compared to only 44.38% in 'Korgut' in comparison to their respective control. No significant change in the *E* was observed in 'Korgut' stressed up to 120 mmol/l NaCl (Fig. 3.5.1 b).

Similar results were also observed for stomatal conductance. 'Jaya' variety showed a linear decrease in *gs*, while 'Korgut' did not change gs until 120 mmol/l NaCl. 'Jaya' showed a decrease of 99% at 200 mmol/l NaCl compared to only 50% seen in 'Korgut' for the same concentration of salt compared to their respective control (Fig. 3.5.1 c).

No significant change in intercellular CO_2 concentration (*Ci*) was observed in both 'Jaya' and 'Korgut' grown with salt stress (Fig. 3.5.1 d), but P_N/Ci ratio was higher in 'Korgut' at high salt stress level. Korgut grown at 200 mmol/l NaCl showed a 38% high P_N/Ci ratio than 'Jaya' for the same salt concentration (Fig. 3.5.1 f).

3.5.2 Light reaction

Initial chlorophyll fluorescence (Fo), indicative of energy transfer within LHCII, did not show any significant change in Fo value in 'Jaya' treated up to 120 mmol/l NaCl; however, Fo increased to 40% in plants grown at 200 mmol/l salt stress compared to its control. 'Korgut,' on the other hand, showed no such increase in the Fo at any concentration of NaCl (Fig. 3.5.2 a).

Maximum fluorescence (F_m) decreased linearly in both varieties in response to the NaCl treatment. It was observed that Fm declined by 36% in 'Jaya' and 29% in 'Korgut' grown at 200 mmol/l NaCl compared to their respective control (Fig. 3.5.2 b).

The maximum efficiency of PS-II (Fv/Fm) did not show any significant change in the Fv/Fm ratio in both the varieties treated up to 120 mmol/l salt stress; however, Fv/Fm

ratio declined to 69% in 'Jaya' compared to only 13% in 'Korgut' treated with 200 mmol/l NaCl as compared to their respective controls (Fig. 3.5.2 c).

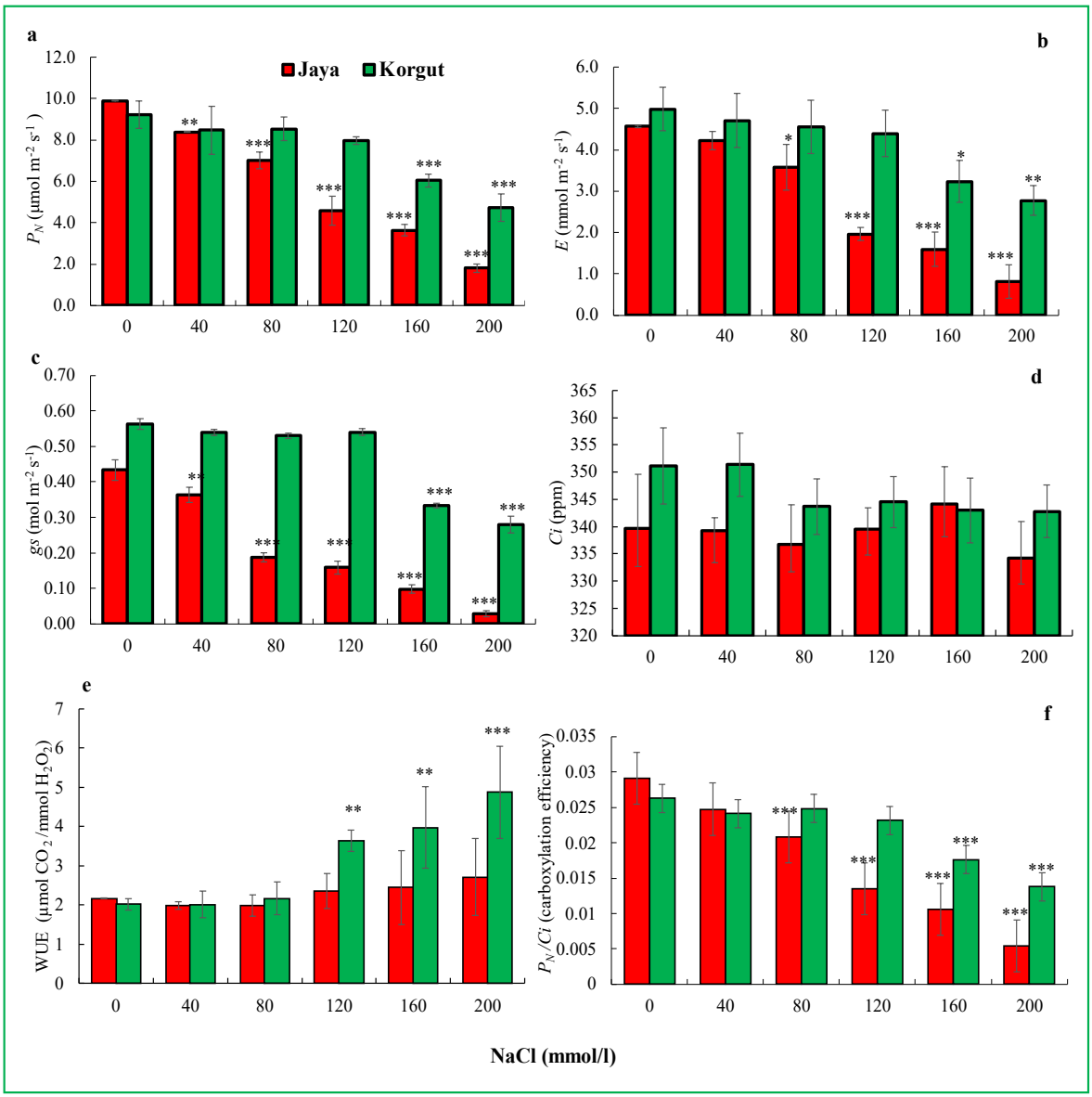


Fig. 3.5.1: Effect of NaCl on (a) net photosynthetic (P_N), (b) transpiration rate (E), (c) stomatal conductance (gs), (d) internal CO_2 concentration (Ci), (e) water use efficiency (WUE), and (f) carboxylation efficiency (P_N/Ci) in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (*), (**), (***) indicates the significance at $p< 0.05$, $p< 0.005$, $p< 0.01$ respectively. Standard deviation (±SD) indicates the means of three replicates.

Our results show a much higher value of qP (0.78) for 'Korgut' than for 'Jaya' (0.41) control. The qP was insignificantly affected at the lower salinity range. It was observed that qP showed no significant change for the 'Jaya' variety up to 80 mmol/l NaCl, while

'Korgut' showed no effect up to 160 mmol/l NaCl as compared to their respective control. Treatment of 200 mmol/l NaCl resulted in a 77% decrease in qP value in 'Jaya,' while only a 15% decrease was observed in 'Korgut' grown at the same salt concentration as their control (Fig. 3.5.2 d).

Non-photochemical quenching (qNP) increased linearly in both the rice varieties with an increase in the salt concentration and showed a significant increase, 116% in 'Jaya' and 157% in 'Korgut,' grown at 200 mmol/l NaCl concentration as compared to their respective control (Fig. 3.5.2 e).

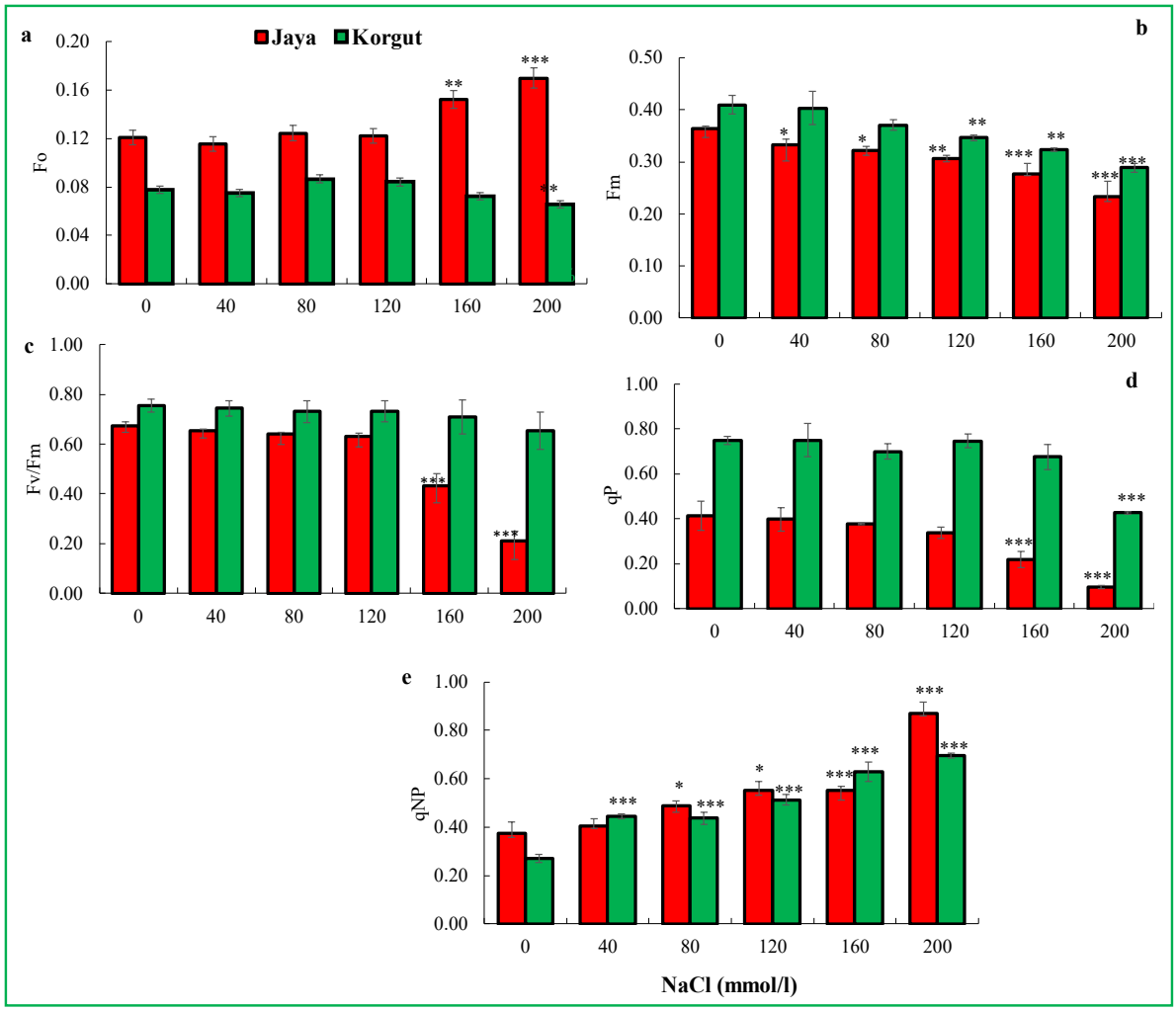


Fig. 3.5.2: Effect of NaCl on (a) initial fluorescence (Fo), (b) maximum fluorescence (Fm), (c) variable fluorescence (Fv/Fm), (d) photochemical quenching (qP), and (e) non-photochemical quenching (qNP) in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (*), (**), (***) significant at $p< 0.05$, $p< 0.005$, $p< 0.01$ respectively. Standard deviation (±SD) indicates the means of three replicates.

3.5.3 Pigment analysis

A qualitative and quantitative profile of photosynthetic pigments in response to NaCl treatment was studied using HPLC. HPLC profile extracted at 445 nm to show qualitative changes (Fig. 3.5.3A, B). It was seen that treatment of both the varieties with salt did not show any qualitative changes in the pigments and showed the presence of neoxanthin, violaxanthin, lutein, and β -carotene as carotenoids and chlorophyll 'a' and chlorophyll 'b' in all the samples (Table 3.5.3). However, quantitative changes in chlorophyll and carotenoid contents were observed as a result of the salt treatment in both varieties. Our data showed a linear decrease in both total chlorophyll and total carotenoids due to an increase in the salt treatment in both varieties. Total chlorophyll decreased by 74%, with 82 and 72% decreases in Chl 'a' and Chl 'b,' respectively, in the 'Jaya' variety grown at 200 mmol/l NaCl. 'Korgut' for the same treatment, however, showed a decrease of 57% in total chlorophyll with 52 and 61% decrease in Chl 'a' and Chl 'b,' respectively. Our results showed that the Chl a/b ratio declined in 'Jaya' but increased in the 'Korgut' variety due to the salt treatment (Table 3.5.3).

Similarly, lutein, neoxanthin, violaxanthin, and β-carotene also decreased to variable levels in both varieties. Total carotenoid content declined to 63% at the highest level of salt stress in the 'Jaya' variety, while the same salt stress decreased by 48% in the 'Korgut' variety compared to their respective control. Carotenoid to chlorophyll ratio (Car/Chl) showed a decrease with increasing salt stress except for plants grown at 200 mmol/l NaCl in the case of the 'Jaya' variety, while 'Korgut' showed an increase in the ratio of Car to Chl in plants stressed at 80 mmol/l and higher concentration of NaCl (Table 3.5.3)

Table 3.5.3: Effect of NaCl on photosynthetic pigments in 'Jaya' and 'Korgut' rice varieties treated for 21d.

Rice Variety	Salt Stress	Pigment content (µg/g FW)									
	(mmol /l)	Chl 'a'	Chl 'b'	Total chlorophyll	a/b	lutein	Neoxanthin	Viloxanthin	b-carotene	Total carotene	Car/Chl
'Jaya'	0#	318.30±20.5	374±13	692.303±33.4	0.841	8.37±1.16	11.19±1.06	22.47±1.50	3.56±0.54	48.76±14	0.070
	40	225.38±17.18	284.6±15.9	510.052±24.3	0.817	5.69±0.80	9.15±0.99	16.50±1.47	4.5±0.55	37.03±3.5	0.072
	80	171.18±53.3	235.6±8.08	406.853±52.6	0.691	4.40±0.77	6.06±1.87	10.59±0.65	5.69±0.91	24.55±9.9	0.076
	120	160.2±23.06	253±38.4	413±17.3	0.730	6.45±1.00	6.05±2.27	8.606±1.87	7.33±0.74	31.69±9.8	0.076
	160	123.2±3.46	192.6±3.97	315.667±6.6	0.701	5.90±1.22	3.87±0.65	3.59±0.85	8.28±0.55	23.00±9.61	0.072
	200	57.66±14.06	101±3.93	158.692±13.5	0.710	4.24±0.99	2.30±0.55	3.29±1.007	8.10±1.7	17.31±4.5	0.109
'Korgut'	0#	327.6±12.42	225±23.09	565.333±20.5	1.389	3.67±0.98	14.57±0.54	17.88±2.30	6.86±0.81	43.85±8.5	0.077
	40	374±11.53	248±9.32	610.667±12	1.582	3.40±0.50	11.19±1.78	22.65±0.77	7.1±0.72	40.36±10.2	0.066
	80	242.6±14.64	126.12±12.6	373.373±18	1.868	2.13±0.43	6.25±1.98	16.08±3.25	8.56±1.15	29.37±12.2	0.078
	120	194.33±6.4	107±2.34	298.333±8.12	1.868	2.42±0.40	6.12±2.37	13.14±1.7	8.466±1.15	26.71±6.9	0.089
	160	167±8.18	84±1.21	250±7.2	2.013	1.97±0.64	4.64±2.30	13.63±1.22	8.9±1.0	24.71±3.4	0.098
	200	158.73±5.5	88±4.23	244.667±1.15	1.857	1.43±0.48	4.58±2.15	12.78±1.38	9.69±0.42	22.38±5.2	0.091

0# (Control, i.e., without NaCl treatment). Standard deviation (±SD) indicates the means of three replicates.

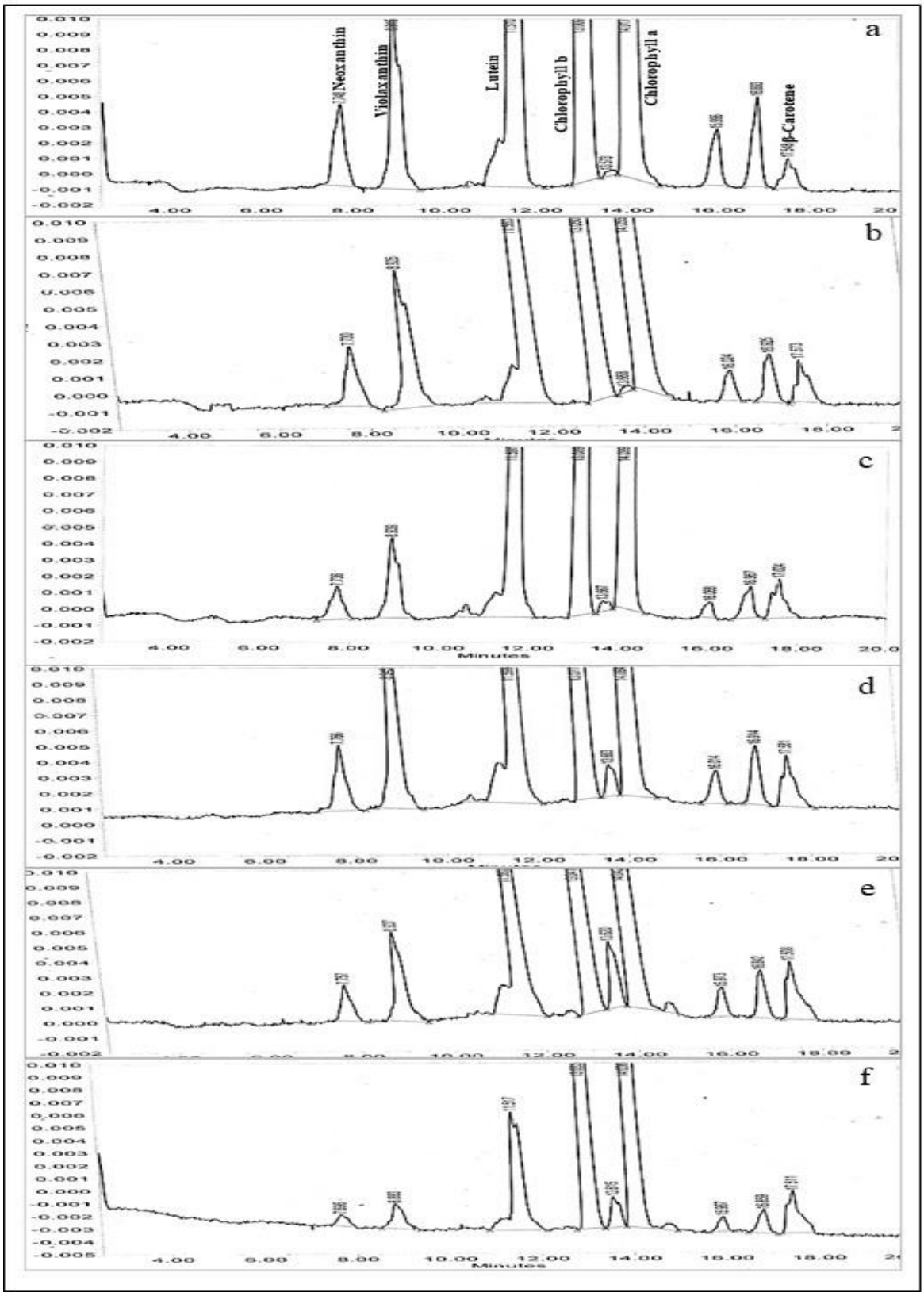


Fig. 3.5.3A: HPLC profile extracted at 445 nm of photosynthetic pigments in 'Jaya' variety of rice treated with NaCl for 21d; control (a), 40 mmol/l (b), 80 mmol/l (c), 120 mmol/l (d), 160 mmol/l (e), and 200 mmol/l (f).

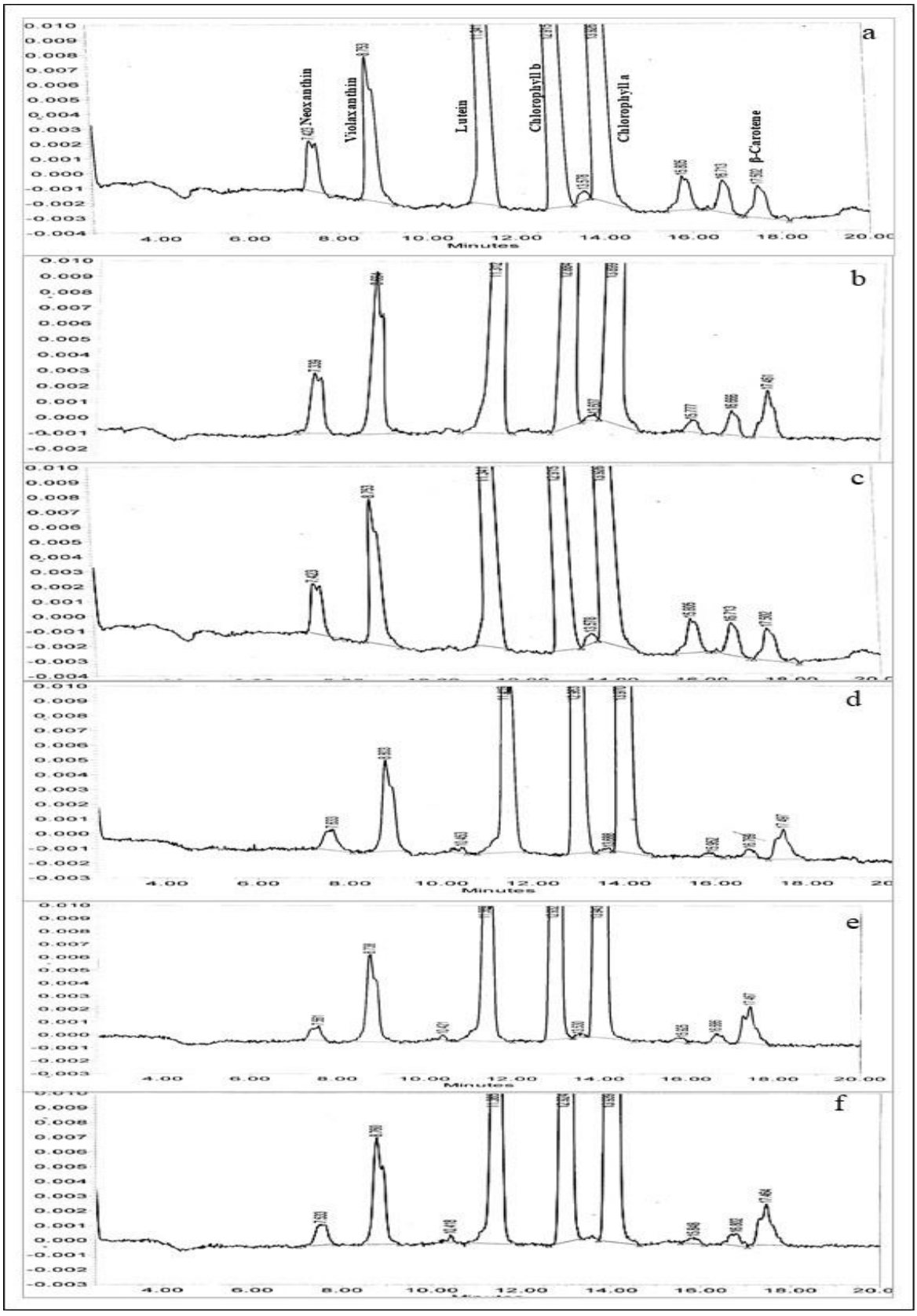


Fig. 3.5.3B: HPLC profile extracted at 445 nm of photosynthetic pigments in 'Korgut' variety of rice treated with NaCl for 21d; control (a), 40 mmol/l (b), 80 mmol/l, (c) 120 mmol/l (d), 160 mmol/l (e), 200 mmol/l (f).

3.6 Effect of salt stress on ROS generation

3.6.1 Hydrogen peroxide (H_2O_2)

A significant increase in H_2O_2 content was observed in both varieties due to the salt stress (Fig. 3.6.1). With an increase in NaCl concentration, there was a progressive increase in the level of H_2O_2 content. As compared to its control, a linear increase of 47, 86, and 168% was seen in 'Jaya' at 40, 120, and 200 mmol/l NaCl treatment. In 'Korgut,' the increase in the H_2O_2 content was 8, 86, and 136%, respectively, for the same three concentrations of NaCl treatment. Results demonstrate 2.7-fold higher H_2O_2 content in 'Jaya' than in 'Korgut' leaves at 200 mmol/l NaCl concentrations (Fig. 3.6.1).

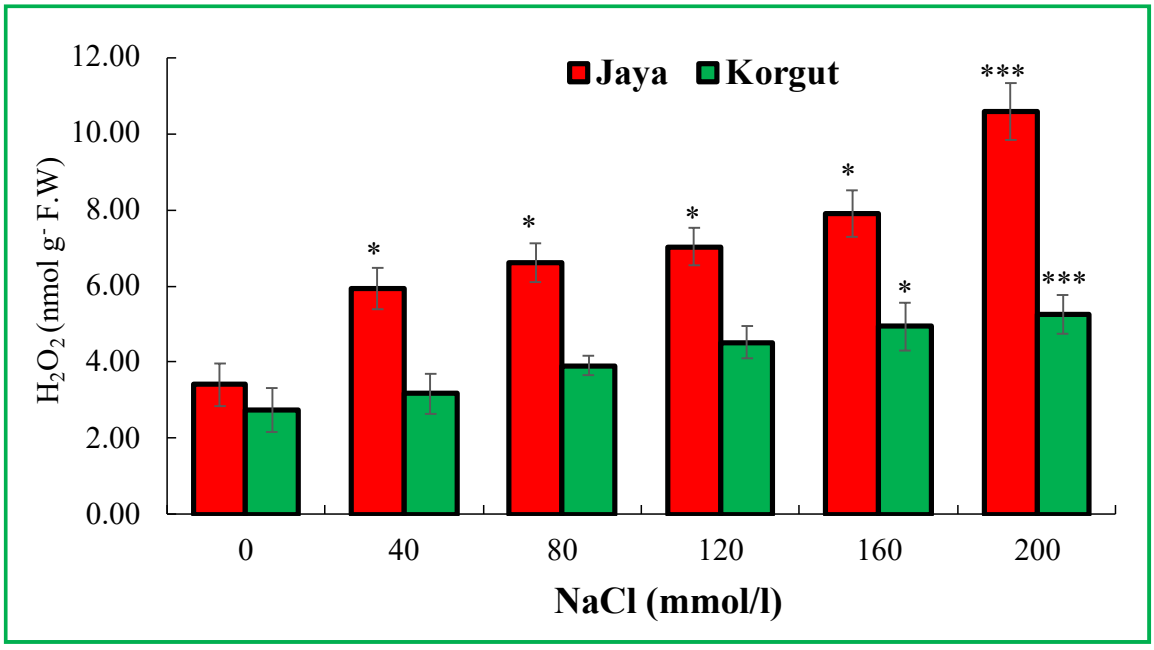


Fig. 3.6.1: Effect of NaCl on hydrogen peroxidation (H_2O_2) in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (*), (**), (***) indicate the mean difference between salt stress response compared to control is significant at $p< 0.05$, $p< 0.005$, $p< 0.01$, respectively. Standard deviation (±SD) indicates the means of three replicates.

3.6.2 Hydroxyl Radicals (OH•)

An increase in OH• also collaborated with the rise in H_2O_2, showing similar patterns of enhancement in response to salt stress in both the studied varieties. As the NaCl concentration increased, there was a progressive rise in the level of OH• radicals in both varieties. A linear increase of 74, 106, and 210% was seen at 40, 120, and 200 mmol/l of NaCl treatment in a 'Jaya' variety compared to its control (Fig. 3.6.2). However, in the 'Korgut' variety, the increase in the OH• radicals was 18, 55, and 75%, respectively, for

the same three concentrations of NaCl treatment as compared to its control. An excess of H_2O_2 positively correlates to an increase in OH• in 'Jaya' (r^2 = 0.9) than observed in 'Korgut' (r^2 = 0.6) (Table 3.9.3). Compared to 'Korgut,' OH• content in 'Jaya' leaves was 2-fold higher at 200 mmol/l of NaCl treatments (Fig. 3.6.2).

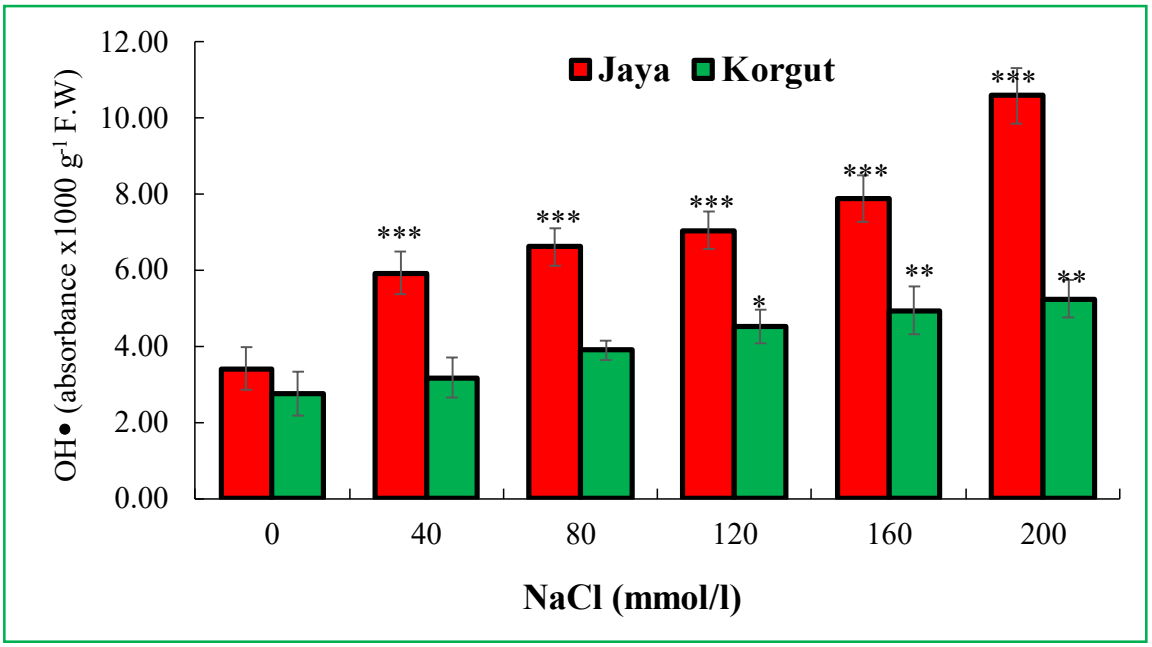


Fig. 3.6.2: Effect of NaCl on hydroxyl radical (OH•) in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (*), (**), (***) indicate the mean difference between salt stress response compared to control is significant at $p< 0.05$, $p< 0.005$, $p< 0.01$, respectively. Standard deviation (±SD) indicates the means of three replicates.

3.7 Oxidative damage

3.7.1 Lipid peroxidation (MDA)

Lipid peroxidation was measured as MDA-TBA content. Throughout the experiment, the content of MDA was higher in 'Jaya' compared to 'Korgut' (Fig. 3.7.1). The MDA content in the 'Jaya' increased gradually to 14, 62, and 270% at 40, 120, and 200 mmol/l NaCl concentration, while in 'Korgut,' an increase of only 13, 44, and 115% in the MDA content were observed at the same concentration of salt in comparisons to their respective control. Compared to 'Jaya,' MDA content in 'Korgut' leaves was 2.4 times lesser at 200 mmol/l NaCl concentration (Fig. 3.7.1).

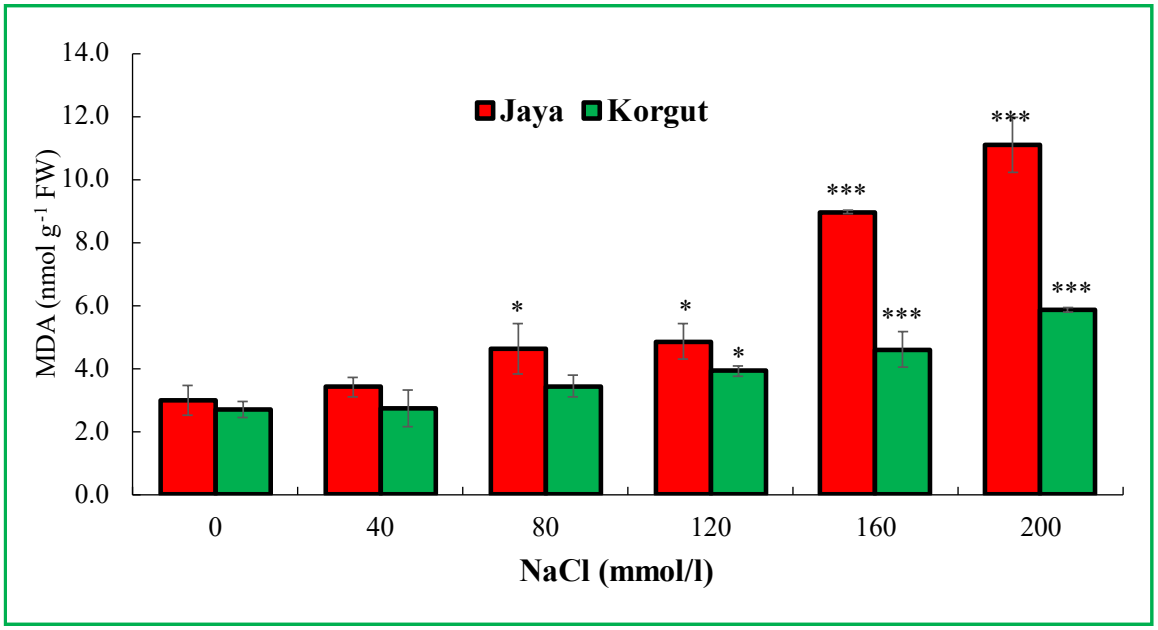


Fig. 3.7.1: Effect of NaCl on lipid peroxidation (MDA) in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (**), (***) indicates the significant at $p< 0.005$, $p< 0.01$ respectively. Standard deviation (±SD) indicates the means of three replicates.

3.7.2 Membrane Stability Index (MSI)

The degree of membrane stability due to salinity is assessed by direct measurement of electric conductivity (EC), an indicator of solute leakage. The solute leakage was much higher in the 'Jaya' than in the 'Korgut' variety. A linear increase of 59, 153, and 213% in electrolyte leakage was seen at 40, 120, and 200 mmol/l of NaCl treatment, respectively, in a 'Jaya' variety compared to its control (Fig. 3.7.2). While in 'Korgut,' the increase in the electrolyte leakage was only 17, 55, and 61% at the same salt concentration as compared to their control. In comparison to 'Korgut,' electrolyte leakage in 'Jaya' leaves was approximately 3.4-fold higher at 200 mmol/l NaCl concentrations (Fig. 3.7.2).

3.7.3 Carbonyl content (CO)

As NaCl concentration increased, there was a progressive increase in the level of carbonyl content (CO) in both the 'Jaya' and 'Korgut' varieties. A linear increase of 39, 112, and 301% in CO was seen at 40, 120, and 200 mmol/l NaCl treatment, respectively, in the 'Jaya' variety, compared to its control. However, in the 'Korgut' variety, an increase of 64, 159, and 218% in CO was observed at the same concentration of NaCl treatment compared to their control (Fig. 3.7.3). In comparison to 'Korgut,' CO content

in 'Jaya' leaves was approximately 1.7-fold higher at 200 mmol/l NaCl concentrations (Fig. 3.7.3).

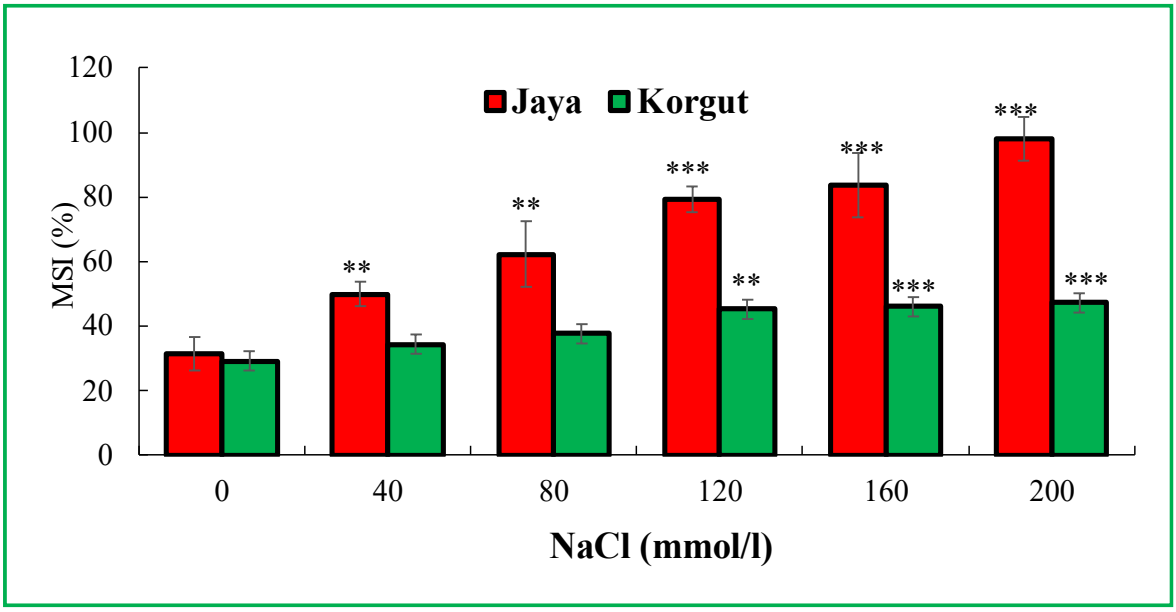


Fig. 3.7.2: Effect of NaCl on membrane stability index (MSI) in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (**), (***) indicates the significant at $p< 0.005$, $p< 0.01$ respectively. Standard deviation (±SD) indicates the means of three replicates.

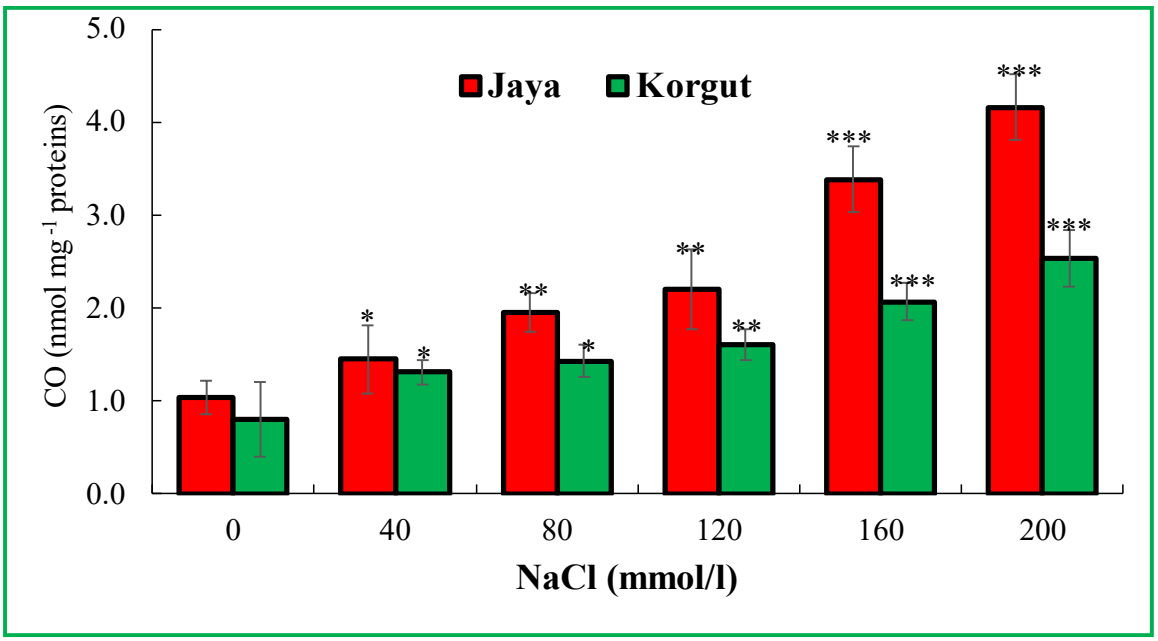


Fig. 3.7.3: Effect of NaCl on carbonyl content (CO) in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (*), (**), (***) indicate the mean difference between salt stress response compared to control is significant at $p< 0.05$, $p< 0.005$, $p< 0.01$, respectively. Standard deviation (±SD) indicates the means of three replicates.

3.8 Effect of salinity on enzymatic and non-enzymatic antioxidant

3.8.1 Superoxide dismutase (SOD)

The SOD activity was much higher in control plants of 'Korgut' and did not significantly increase on account of salt stress, whereas 'Jaya' showed a linear increase in the SOD activity with increasing concentration of salt treatment; however, the increase in the SOD in 'Jaya' still remained less than what was seen in control of 'Korgut.' The threshold of SOD activity of the 'Korgut' variety in its control plants was 112% higher than seen in the control plants of the 'Jaya' variety. With the rise in NaCl concentration, there was a progressive increase in the SOD activity in the 'Jaya' variety to 6, 25, and 68% at 80, 120, and 200 mmol/l NaCl treatment, respectively, compared to its control. However, in the 'Korgut' variety, SOD activity remained steady with the increasing NaCl treatment and remained 28.4% higher at the highest salt concentration than seen in 'Jaya' (Fig. 3.8.1).

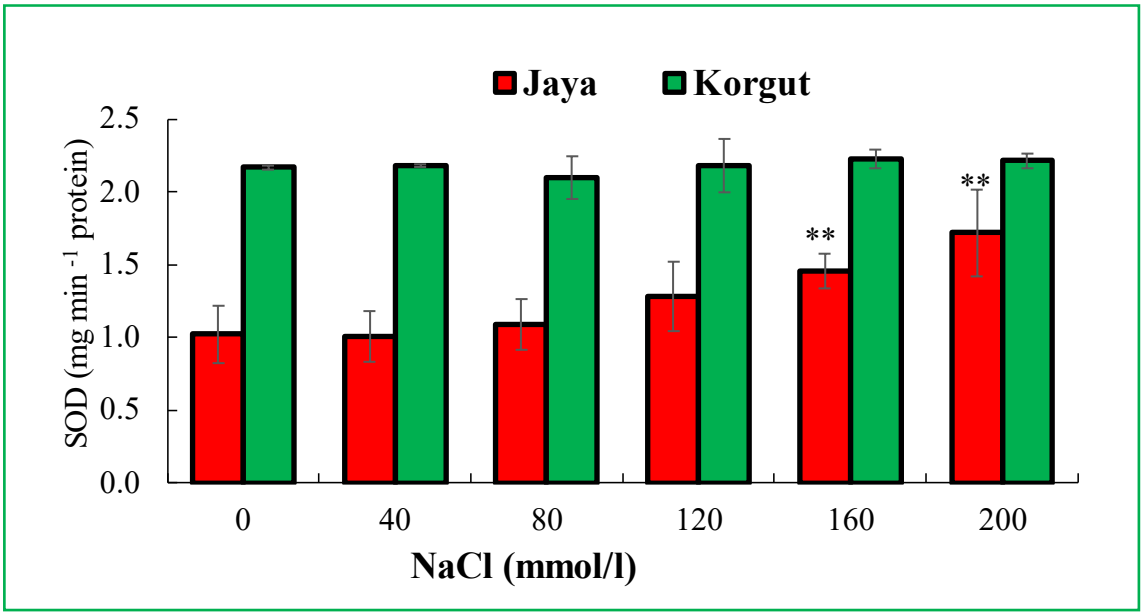


Fig. 3.8.1: Effect of NaCl on superoxide dismutase (SOD) in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (*), (**) indicates the significant at $p< 0.05$, $p< 0.005$ respectively. Standard deviation (±SD) indicates the means of three replicates.

3.8.2 Catalase activity (CAT)

The activity of CAT decreased linearly with increased NaCl concentration in the salt-sensitive 'Jaya' variety but increased in the salt tolerance 'Korgut' variety. A decrease of

13, 26, and 36% was seen at 40, 120, and 200 mmol/l NaCl treatment, respectively, in a 'Jaya' variety, compared to its control.

'Korgut' variety, on the other hand, showed increased CAT activity to 17, 38, and 60% for the same three salt concentrations of NaCl compared to their control. In comparison to 'Jaya,' CAT content in 'Korgut' leaves was 1.8-fold higher at 200 mmol/l NaCl concentrations. (Fig. 3.8.2).

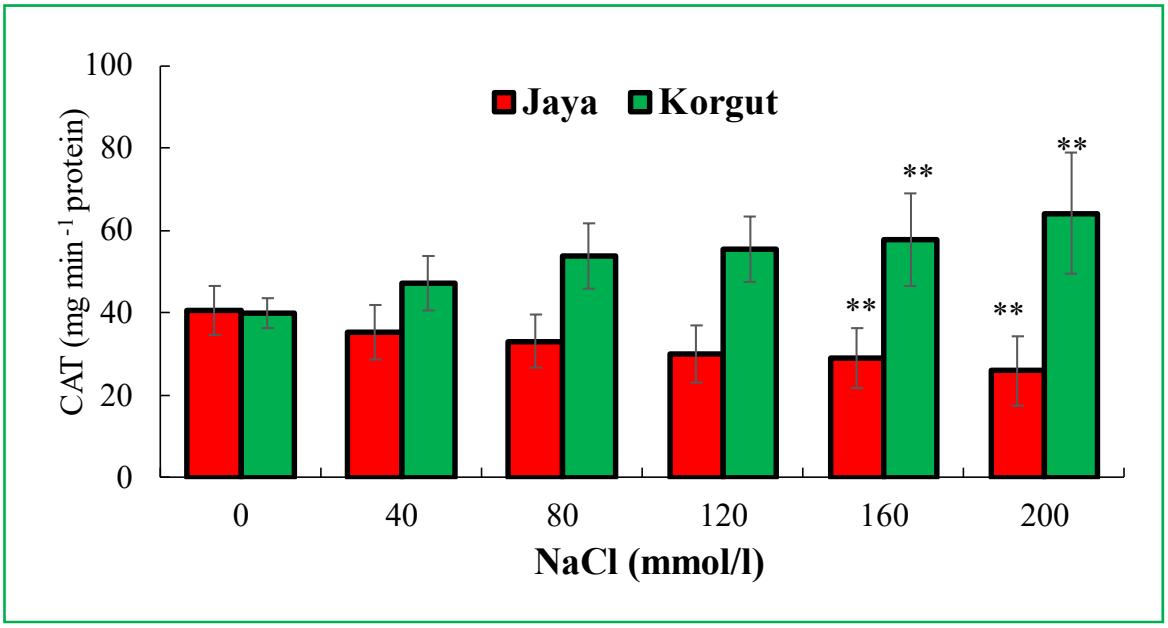


Fig. 3.8.2: Effect of NaCl on catalase (CAT) activity in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (*), (**) indicates the significant at $p< 0.05$, $p< 0.005$ respectively. Standard deviation (±SD) indicates the means of three replicates.

3.8.3 Ascorbate peroxidase (APX)

APX activity showed a 6-fold increase in control 'Korgut' leaves than seen in control 'Jaya'. The salt treatment resulted in an increase in APX activity in both the 'Jaya' and 'Korgut' varieties. The activity of APX linearly increased to 132, and 286% at 120, and 200 mmol/l of NaCl treatment, respectively, in a 'Jaya' variety of rice plants compared to its control. In the 'Korgut' variety, the APX activity increases to 51, and 530% at the same salt concentration of NaCl compared to its control (Fig. 3.8.3). In comparison to 'Jaya,' APX content in 'Korgut' leaves was 10 times higher at 200 mmol/l NaCl concentrations (Fig. 3.8.3).

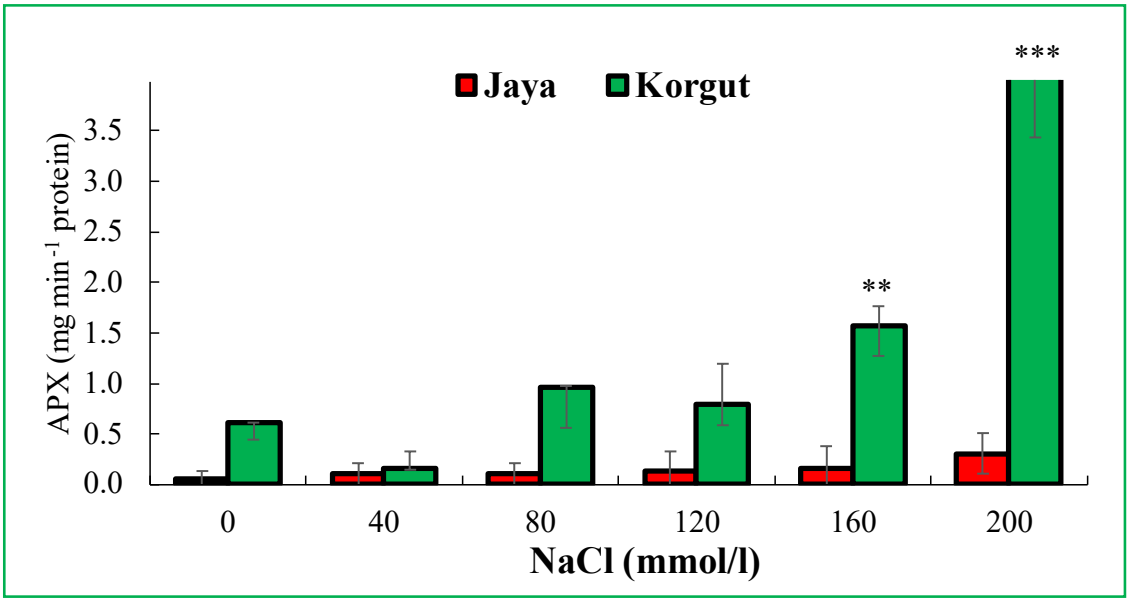


Fig. 3.8.3: Effect of NaCl on ascorbate peroxidase (APX) activity in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (*), (**) indicates the significant at $p< 0.05$, $p< 0.005$ respectively. Standard deviation (±SD) indicates the means of three replicates.

3.8.4 Ascorbic acid (AsA)

Ascorbate content in the control leaves of the 'Korgut' variety was found to be almost double that seen in the control leaves of the 'Jaya' variety. An increase in AsA was observed in both varieties due to salt stress. In the 'Jaya' variety, an increase of 35, 77 and 85% in ascorbate content were seen at 40, 120, and 200 mmol/l NaCl treatment, respectively, as compared to its control, whereas in the 'Korgut' variety, only a slight increase of 4, 6 and 8% were observed for the same salt concentration in comparing to control. However, AsA content was 25% higher at 200 mmol/l NaCl in 'Korgut' than observed in 'Jaya' (Fig. 3.8.4).

3.8.5 Proline content

A significant increase in proline content was also observed in both rice varieties due to the salt stress (Fig. 3.8.5). A linear increase of 2, 6, and 15-fold in proline was seen at 40, 120, and 200 mmol/l NaCl treatment, respectively, in the 'Jaya' variety of rice plants compared to its control. Proline content in the 'Korgut' variety increased sharply with increasing salt stress by 2, 30, and 34-fold for the same concentration of NaCl treatment compared to their control. In addition, in comparison to the 'Jaya' variety, proline content in 'Korgut' leaves was 3-fold lesser at 200 mmol/l NaCl concentration (Fig. 3.8.5).

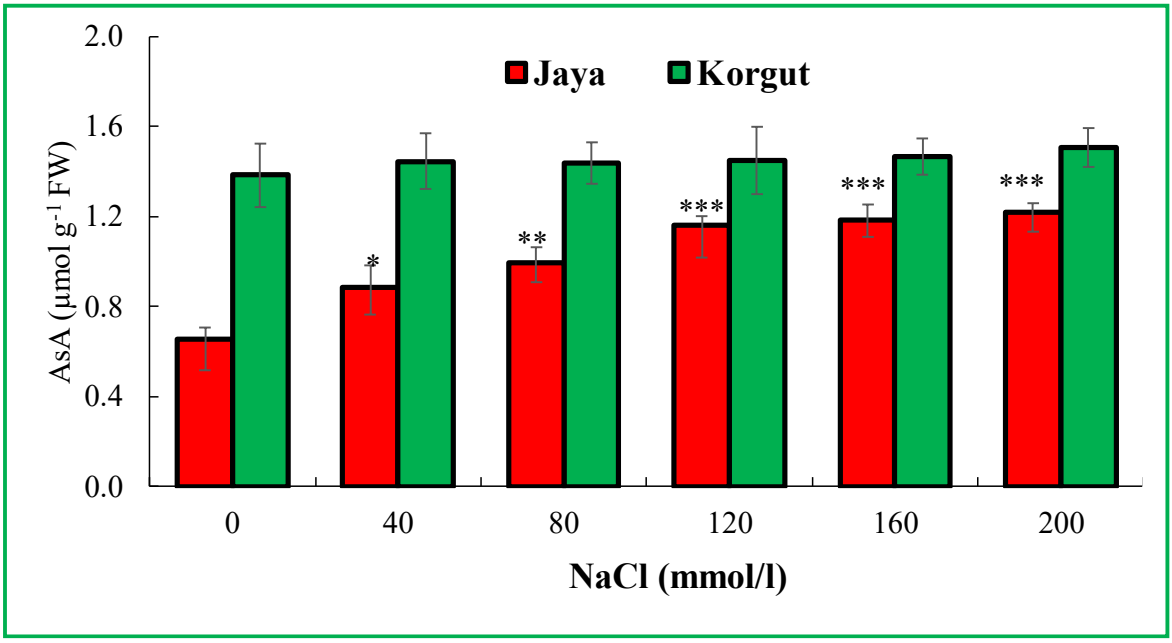


Fig. 3.8.4: Effect of NaCl on ascorbate content (AsA) in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (*), (**), (***) indicates the significant at $p< 0.05$, $p< 0.005$, $p< 0.01$, respectively. Standard deviation (±SD) indicates the means of three replicates.

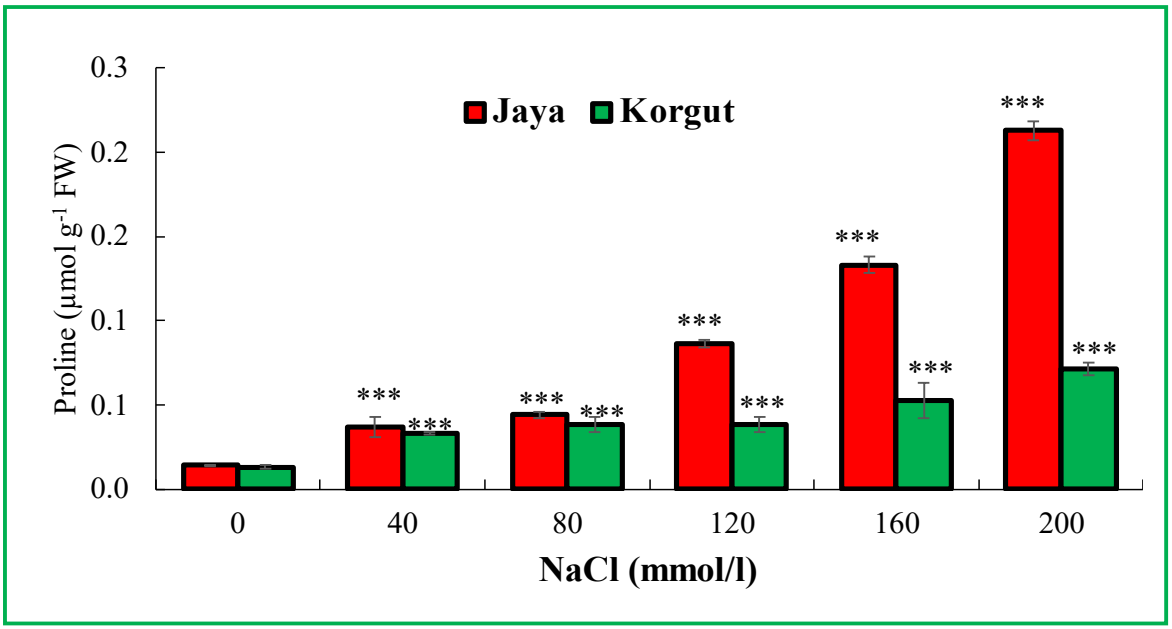


Fig. 3.8.5: Effect of NaCl on proline content in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (***) indicates the significance at $p< 0.01$. Standard deviation (±SD) indicates the means of three replicates.

3.9 Effect of salt stress on lipid analysis (GC-MS)

GC-MS data showed that a total of 9 fatty acids, five saturated, palmitic acid, stearic acid, myristic acid, lauric acid, pentadecanoic acid, and four unsaturated oleic acid, linoleic acid, linoleic acid, and α-linolenic acid, were identified in both the varieties and salt treatment did not cause any qualitative changes in the fatty acid composition in either of rice variety.

Lipid analysis showed that lauric acid and stearic acid decreased with an increasing salt concentration in the 'Jaya' variety. At 200 mmol/l, NaCl treatment resulted in a 48% decrease in lauric and 64% in stearic acid compared to its control. Myristic acid and palmitic acid showed a slight increase in plants treated up to 120 mmol/l NaCl, but a further increase in the NaCl concentrations decreased their content. At 200 mmol/l, NaCl treatment of the 'Jaya' variety resulted in a 41% decrease in myristic acid and 83% in palmitic acid (Table 3.9.1).

The polyunsaturated fatty acids oleic acid and gadoleic acid showed a decrease in their content with increased salt stress in the 'Jaya' variety. 200 mmol/l NaCl treatments to decrease the oleic and gadoleic acid content by 40 and 65%, respectively, compared to its control plants of 'Jaya'. Linolenic acid increases in 'Jaya' treated up to 160 mmol/l NaCl but decreased by 40% when treated with 200 mmol/l NaCl. α-linolenic acid was seen in the 'Jaya' variety control but was not detected in salt stress treatment.

However, the poly/sat ratio decreased as NaCl concentration increased. At 200 mmol/l, NaCl treatment resulted in a 5% decrease in the pol/sat ratio compared to the control (Table 3.9.1). Irrespective of changes in saturated and unsaturated fatty acid, total fatty acid declined due to salt treatment in the 'Jaya' variety.

Lipid analysis showed that lauric acid and myristic acid decreased with increased salt concentration in the 'Korgut' variety. 200 mmol/l NaCl treatment resulted in a 63% decrease in lauric and 45% in myristic acid compared to its control. Stearic acid showed a slight increase in plants treated up to 120 mmol/l NaCl, but increased NaCl concentrations decreased their content. The 200 mmol/l NaCl treatment of the 'Korgut' variety resulted in a 56% decrease in stearic acid. In contrast, palmitic acid and pentadecanoic acid increased by 54% and 63% at the highest concentration in 'Korgut' compared to the control (Table 3.9.2).

The polyunsaturated fatty acid oleic acid showed a slight decrease in 'Korgut' treated up to 80 mmol/l NaCl, but a further increase in the NaCl concentrations increased their content. At 200 mmol/l NaCl, treatment of the 'Korgut' variety resulted in a 150%

increase in oleic acid compared to the control. Linolenic acid showed a slight rise in 'Korgut' treated up to 80 mmol/l NaCl, but a further decrease in the NaCl concentrations increased their content. At 200 mmol/l, NaCl treatment of the 'Korgut' variety resulted in a 25% decrease in linolenic acid compared to the control. At the same time, gadoleic acid decreased its content with increased salt stress in the 'Korgut' variety. At 200 mmol/l, NaCl treatments decreased gadoleic acid content by 61% compared to its control plants of 'Korgut'.

However, the polyunsaturated and saturated ratio was increased as NaCl concentration increased in the 'Korgut' variety. Treatment of 200 mmol/l NaCl resulted in a 32% increase in the pol/sat ratio compared to the control (Table 3.9.2).

In addition, Pearson correlation analysis was performed among fatty acids and different oxidative stress in the leaves of both varieties (Table 3.9.3). The data revealed that the presence of oleic acid (18:1ω9) and α -linolenic acid had a highly significant and positive correlation with the corresponding parameters, such as H_2O_2 content (r^2 = 0.50, 0.53), OH• production (r^2 = 0.66, 0.56), CO content (r^2 = 0.63, 0.59), and MSI (r^2 = 0.8, 0.59), suggesting that presence of these fatty acid has an imperative role to less oxidative stress in salt tolerance 'Korgut' variety. In addition, the data H_2O_2, OH• CO content, and MSI were negatively correlated with these fatty acids (18:1ω9 and 18:3ω3) in the salt-sensitive 'Jaya' variety (Table 3.9.3).

Table 3.9.1: Fatty acid (FA) composition and their content expressed as mg/g FW in leaves of 'Jaya' (salt-sensitive) variety treated with NaCl for 21d.

'Jaya' variety (mg/g FW)							
S. No.	**Fatty acid**	**0# mmol/l**	**40 mmol/l**	**80 mmol/l**	**120 mmol/l**	**160 mmol/l**	**200 mmol/l**
1	Lauric acid (C12:0)	37.23±3.6	12.86±1.24	33.5±0.98	32.5±0.5	23.0±1.03	19.08±0.8
2	Myristic acid (C14:0)	20.53±0.47	22.03±0.51	24.5±0	51.9±6.175	11.4±1.499	12±0.56
3	Pentadecanoic acid (C15:0)	12.34±0.21	13.34±0.24	14.34±0.21	9.34±0.10	10.34±0	8.34±0
4	Palmitic acid (C16:0)	0.24±0.02	0.637±0.02	0.66±0.05	0.796±0.052	0.122±0.001	0.137±0.001
5	Stearic acid (C 18:0)	0.826±0.041	0.75±0.021	0.736±0.04	1.30±0.06	0.50±0.01	0.82±0.032
6	Oleic acid (C18:1) n-6	1.553±0.26	0.88±0.61	0.437±0.009	0.43±0	0.48±0.03	0.253±0.178
7	Linoleic acid (C18:2) n-6	0.28±0	0.29±0.032	0.37±0.024	0.74±1.11E-16	0.79±0	0.1±1.39E-17
8	α-Linolenic acid (C18:3) n-3	0.044±0.07	Nd	Nd	Nd	Nd	Nd
9	Gadoleic acid (C20:1) n-9	9.367±0.65	6±0.355	8.36±0.323	12.3±1.23	1.256±0.07	6.16±0.04
10	Saturated acid	71.8±15.2	49.2±8.9	73.393±14.23	95.53±21.69	45.793±9.4	40.7±8.01
11	Polyunsaturated acid	11.25 ±3.82	7.80±1.56	6.17±1.75	13.53±5.53	2±0.1	6±1.82
12	Pol/Sat	0.15668±0.01	0.1585±0.0	0.0840±0.01	0.14163±0.0	0.04367±0.0	0.1474±0.0
13	Total fatty acid	83.94±7.53	57.31±6.7	79.5±6.46	109.76±9.58	47.34±4.5	46±2.34

Nd: not detected .0# (Control, without NaCl treatment). Standard deviation (±SD) indicates the means of three replicates.

Table 3.9.2: Fatty acids (FA) composition and their content expressed as mg/g FW in the 'Korgut' variety treated with NaCl for 21 d.

'Korgut' variety (mg/g FW)							
S. No.	**Fatty acid**	**0# mmol/l**	**40 mmol/l**	**80 mmol/l**	**120 mmol/l**	**160 mmol/l**	**200 mmol/l**
1	Lauric acid (C12:0)	55.2±7.11	9.01±0.02	24.2±0.02	12±0.01	19.4±0.3	20.30.02
2	Myristic acid (C14:0)	19.1±0.01	8.6±0.02	7.9±0.89	4.5±0.004	5.4±0.001	10.39±0.21
3	Pentadecanoic (C15:0)	15.21±0	15.26±0.00	14.3±1.8E-15	10.34±0.00	22.32±3.5	24.89±1.23
4	Palmitic acid (C16:0)	0.26±0.014	0.443±0.009	0.44±0.004	0.39±5.55E	0.4±5.6E-17	0.41±0.014
5	Stearic acid (C 18:0)	1.18±0.00	1.216±0.009	1.5±0.042	0.403±0.01	0.503±0.07	0.51±0.014
6	Oleic acid (C18:1) n-9	3.11±0.008	2.073±0.023	2.076±0.0097	10.6±0.28	7.66±0.28	7.8±0.27
7	Linolenic acid(C18:2) n-9	0.229±0.01	0.824±0.02	0.994±0.03	0.20±0.044	0.18±0.047	0.17±0.014
8	α-Linolenic acid (C18:3) n-3	0.746±0.023	0.282±0.02	0.5033±0.03	0.6033±0.10	0.383±0.24	0.767±0.22
9	Gadoleic acid (C20:1) n-9	9.4±0.23	1.96±1.2	2.945±1.77	2.254±1.33	2.28±1.37	6.92±0.77
10	Saturated fatty acid	84.85±22.3	31.93±6.03	47.43±9.7	37.63±5.5	53.62±10.2	62.11±11.1
11	Polyunsaturated fatty acid	13.88±3.7	5.142±0.75	6.466±0.94	13.66±3.2	10.46±2.4	15.6±3.6
12	Pol/Sat	0.1636±0.02	0.167±0.03	0.1475±0.01.2	0.369±0.021	0.1973±0.01	0.2616±0.013
13	Total fatty acid	98.73±15.2	36.02±19.17	53.93±19.12	50.29±19.8	63.07±13.2	77.77±14.23

0# (Control, without NaCl treatment). Standard deviation (±SD) indicates the means of three replicates.

Table 3.9.3: Pearson correlation analysis between hydrogen peroxide (H_2O_2), hydroxyl radicals (OH•), membrane stability (MSI), carbonyl content (CO), and unsaturated fatty acid, oleic acid (18:1ω9) and α- linolenic acid (18:3ω3).

'Korgut'	H_2O_2	OH•	CO	MSI	18:1ω9	18:3ω3
H_2O_2	1	0.620**	0.910**	0.712**	0.500*	0.532*
OH*	0.620**	1	0.760**	0.853**	0.661**	0.563*
CO	0.910**	0.760**	1	0.869**	0.636**	0.595**
MSI	0.712**	0.853**	0.869**	1	.818**	0.595**
18:1ω9	0.500*	0.661**	.636**	0.818**	1	0.483*
18:3ω3	0.532*	0.563*	0.595**	0.595**	0.483*	1
'Jaya'						
H_2O_2	1	0.905**	0.797**	0.801**	-0.701**	-0.553*
OH*	0.905**	1	0.895**	0.883**	-0.829**	-0.715**
MSI	0.797**	0.895**	1	0.874**	-0.799**	-0.713**
CO	0.801**	.883**	0.874**	1	-0.657**	-0.529*
18:1ω9	-0.701**	-0.829**	-0.799**	-0.657**	1	0.902**
18:3ω3	-0.553*	-0.715**	-0.713**	-0.529*	0.902**	1

** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

3.10 Effect of salt stress on thylakoid membrane proteins

In our study, a total of 12 distinct bands were observed in both varieties without any qualitative changes due to the treatment (Fig. 3.10.1 and 3.10.2). The mass of some of the proteins bands was identified based on marker protein and found to be 63-65kDa chloroplast coupling factor (CF1), 47 & 43kDa protein, chlorophyll protein CP47, and CP43, 32-33kDa molecular weights corresponding to D1and D2 protein, 20-28kDa of LHC and 10-11kDa of Cyt_b559 protein. Salt treatment to the 'Jaya' variety showed significant quantitative changes in their thylakoid membrane proteins profile than in the 'Korgut' variety. Plants of the 'Jaya' variety grown up to 120 mmol/l NaCl showed no significant quantitative change in the protein profile; however, higher than 120 mmol/l NaCl caused considerable loss of protein in the range of <10kDa (Cyt_b559), 28-24kDa (OEC), 32kDa (D1-D2) and 43-63kDa (LHC; Fig. 3.10.1). However, the 'Korgut' variety did not show any such significant decrease in the protein profile of the thylakoid membrane (Fig. 3.10.2).

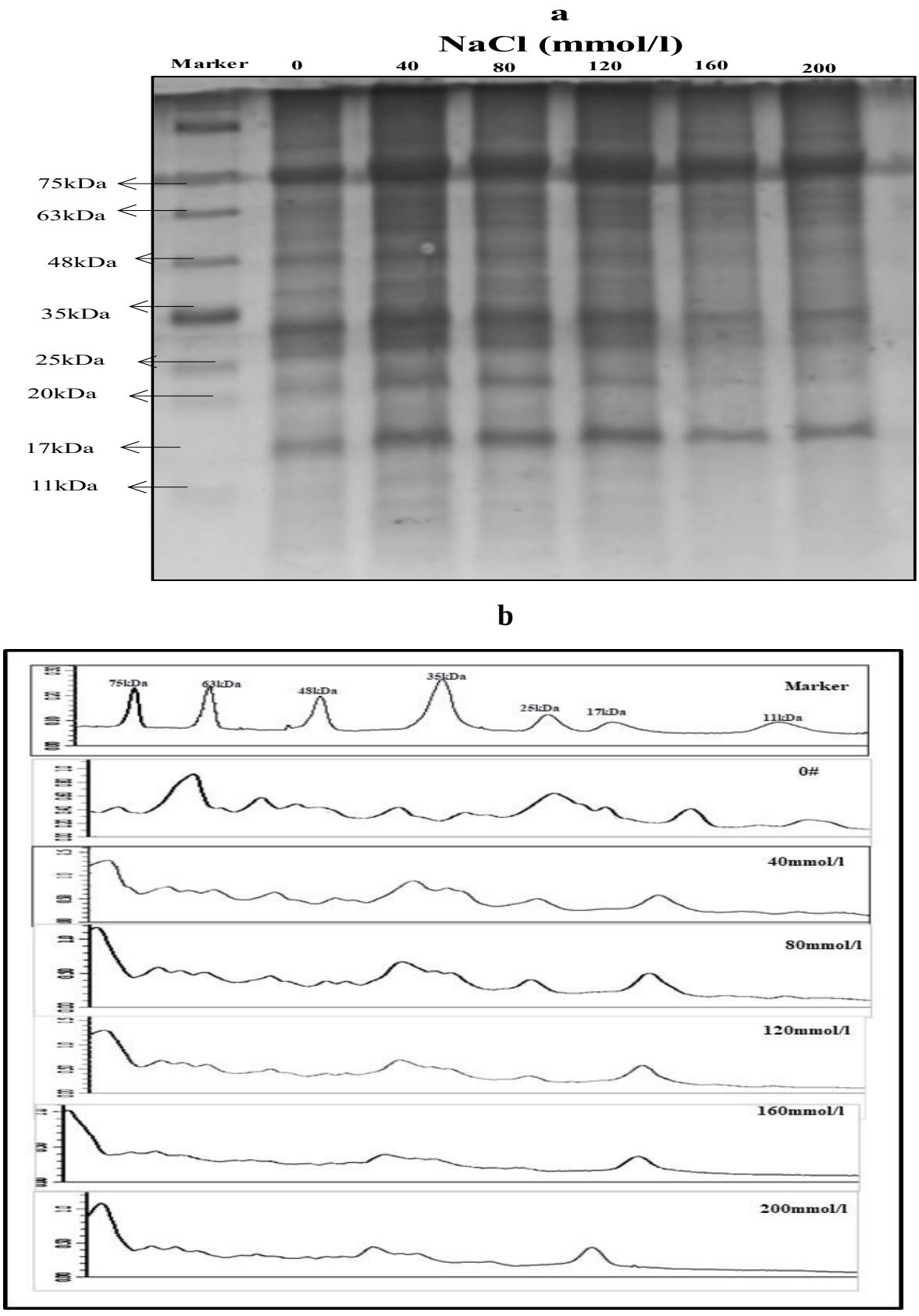


Fig. 3.10.1 Electrophoresis profile of thylakoid membranes from leaves of 'Jaya' variety treated with 0-200 mmol/l NaCl (a), optical density scan of the SDS-PAGE (b).

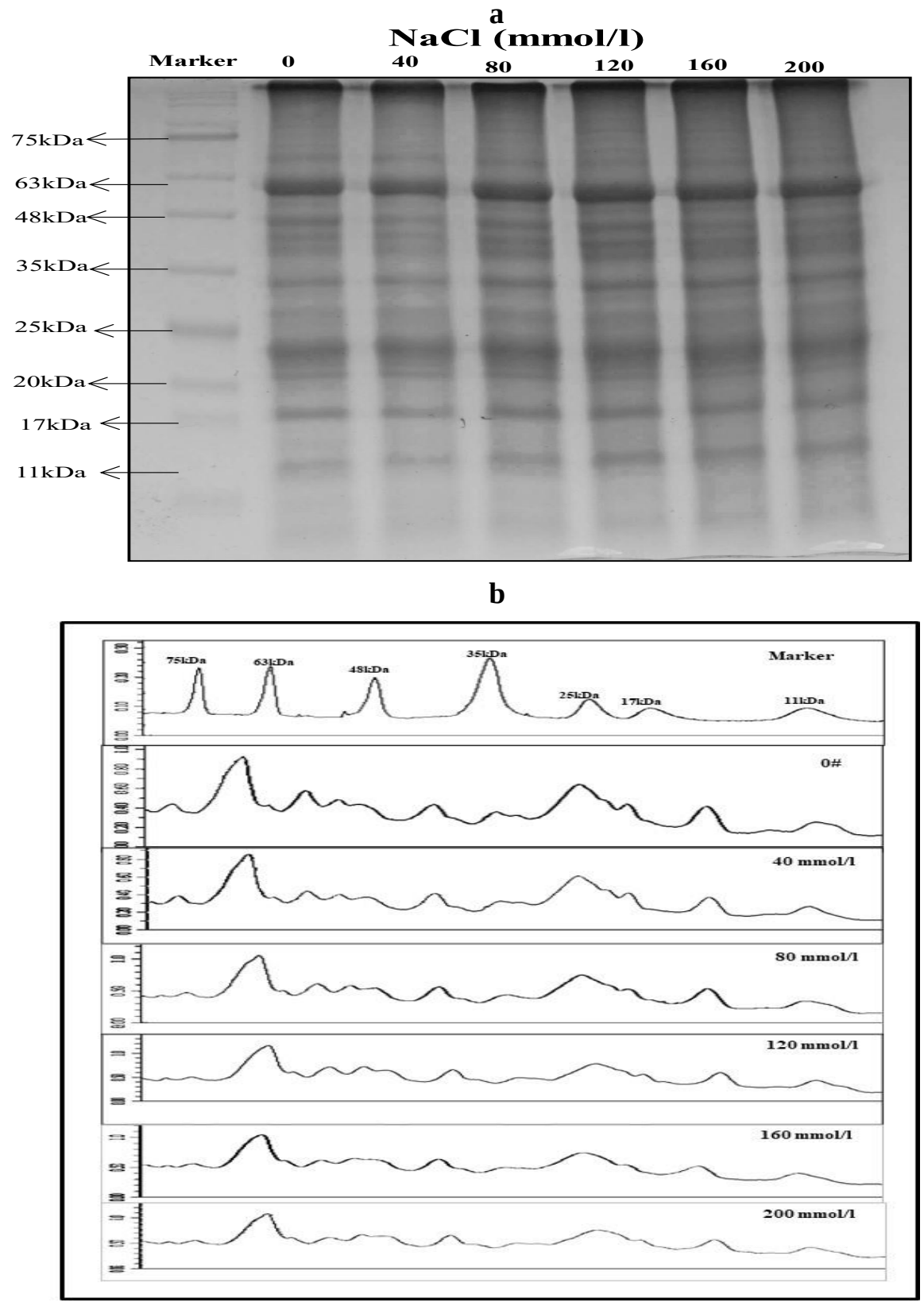


Fig. 3.10.2 Electrophoresis profile of thylakoid membranes from leaves of 'Korgut' variety treated with 0-200 mmol/l NaCl (a), optical density scan of the SDS-PAGE (b).

3.11 Effect of salt stress on the protein profile of rice leaves

The proteomic analysis used SWATH, separating a total protein of 958 in both varieties (Fig. 3.11.1A, B). Out of 958, only 520 proteins were identified, 248 proteins were seen at two concentrations (control and 200 mmol/l NaCl), known as matched, and 272 proteins were seen at one concentration or the other but not at both the concentration, known as unmatched in 'Jaya' variety. Out of 248 matched proteins, 46 showed upregulation, 48 showed downregulation, whereas 154 proteins showed insignificant changes of less than one-fold (Fig. 3.11.2a and Fig. 3.11.3). However, in the 'Korgut' variety, the proteomic analysis resulted in 341separation of common proteins (control and 200mmol/l NaCl) known as matched, and 179 proteins were seen at one concentration or the other but not at both the concentration known as unmatched. Out of 341 matched proteins, 86 showed upregulation, and 77 showed downregulation, whereas 178 proteins showed insignificant changes of less than one-fold (Fig. 3.11.2a and Fig. 3.11.3). In 'Korgut,' most proteins are more than 2-fold compared to 'Jaya' and show the varietal difference (Fig. 3.11.2 b). All the downregulated and upregulated proteins are listed in Table 3.11.1 and categorized based on gene ontology (GO) (Fig. 3.11.4 and 3.11.5).

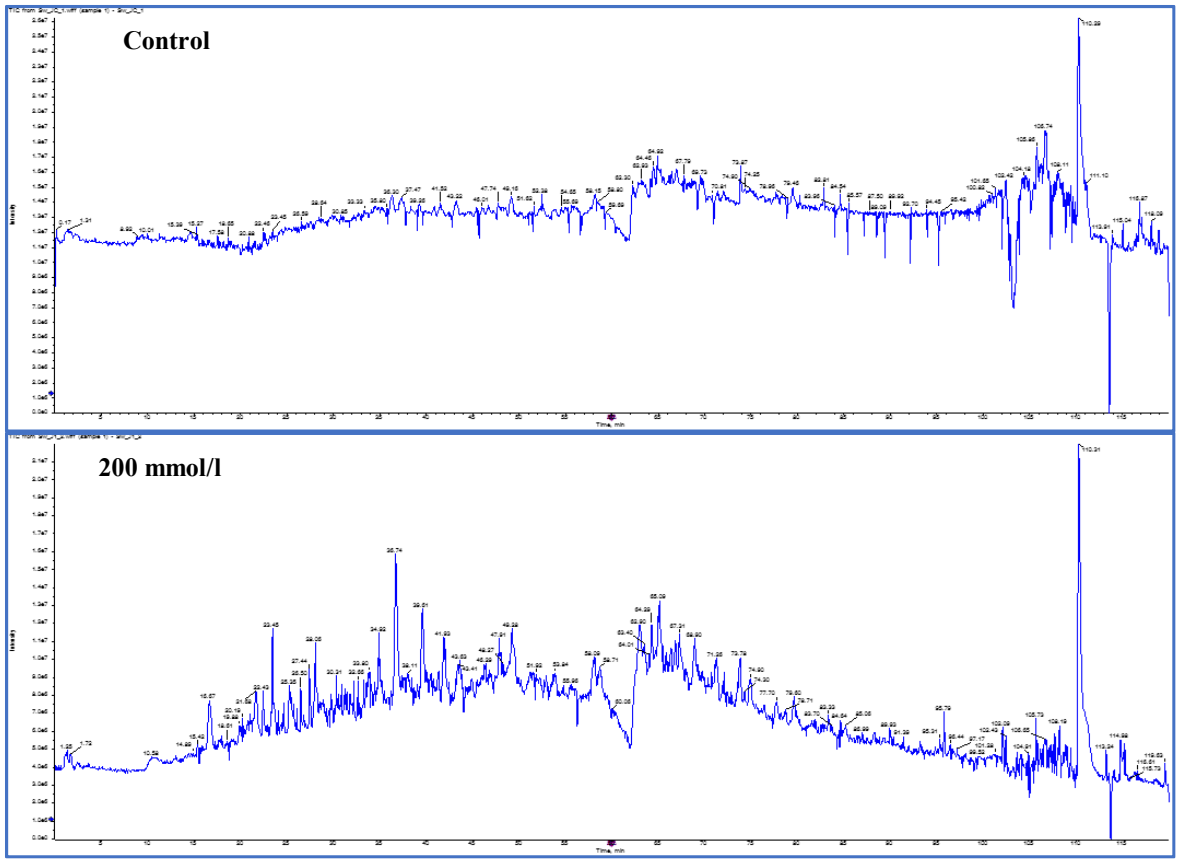


Fig. 3.11.1A: LC-MS peptide map profile of 'Jaya' variety treated with NaCl for 21d.

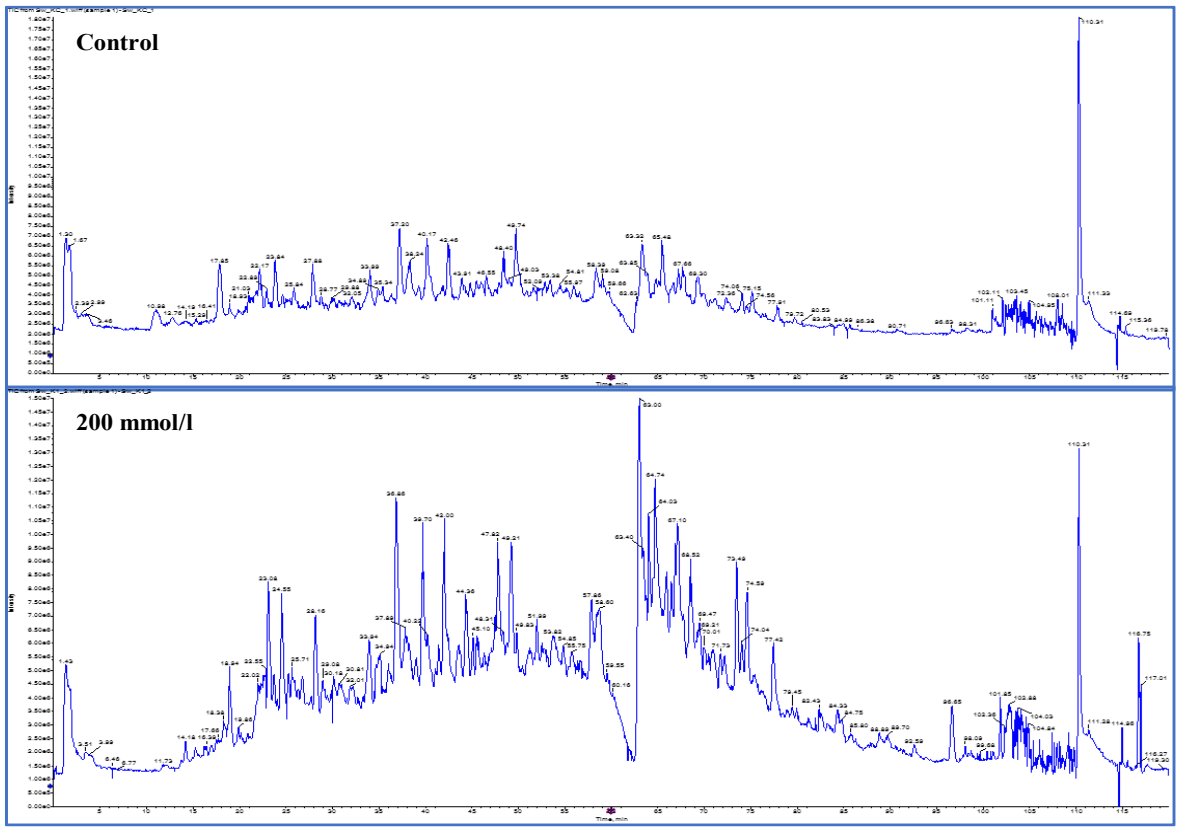


Fig. 3.11.1B: LC-MS peptide map profile of 'Korgut' variety treated with NaCl for 21d.

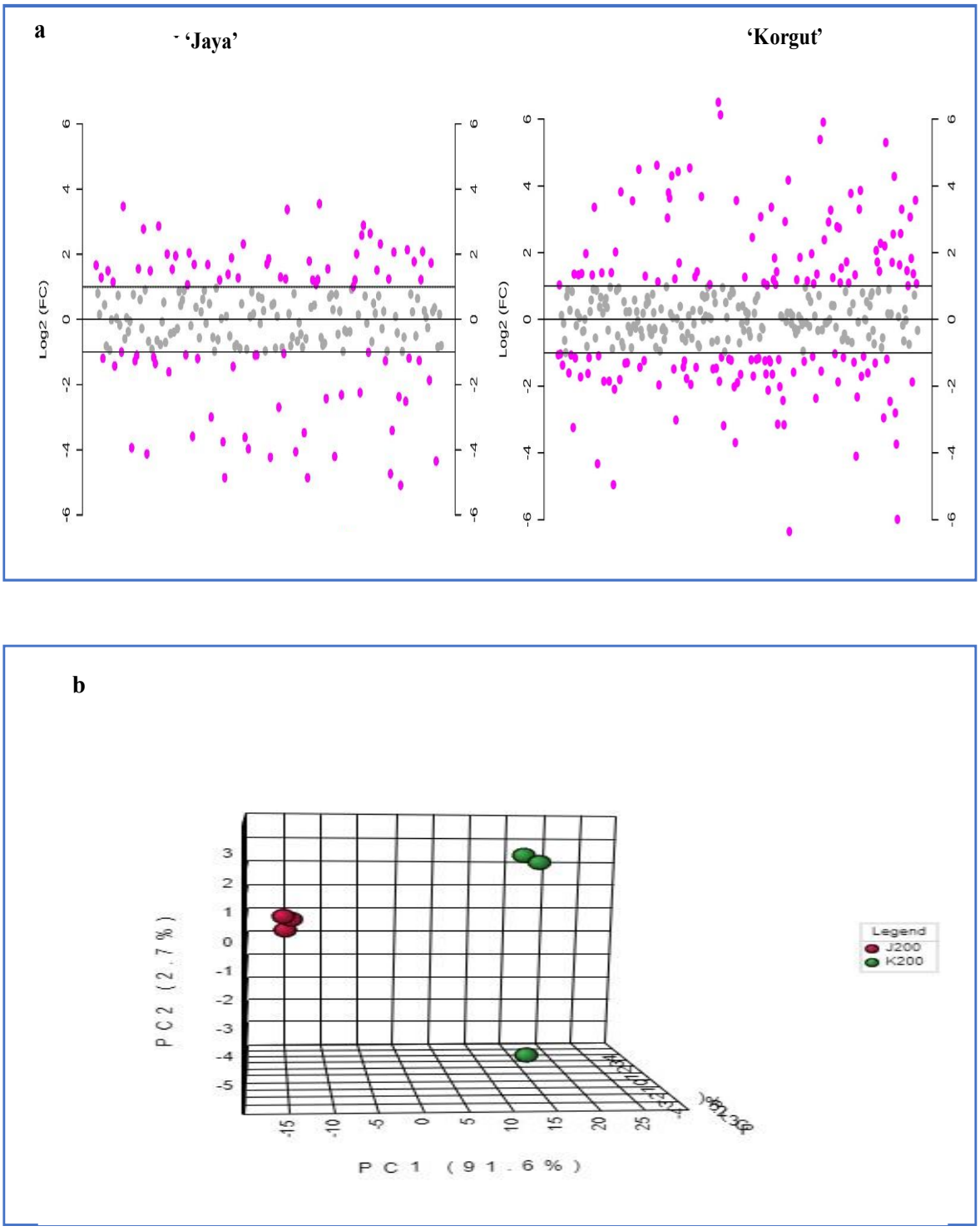


Fig. 3.11.2: Volcano Plot of 'Jaya' and 'Korgut' showing Log ± 2 fold changes in a number of proteins (purple) and unchanged proteins (gray) at 200 mmol/l as compared to its control (a), and PCA plot of 'Jaya' (red) and 'Korgut' (green) showing the varietal difference (b). Points of each cluster show the replicates.

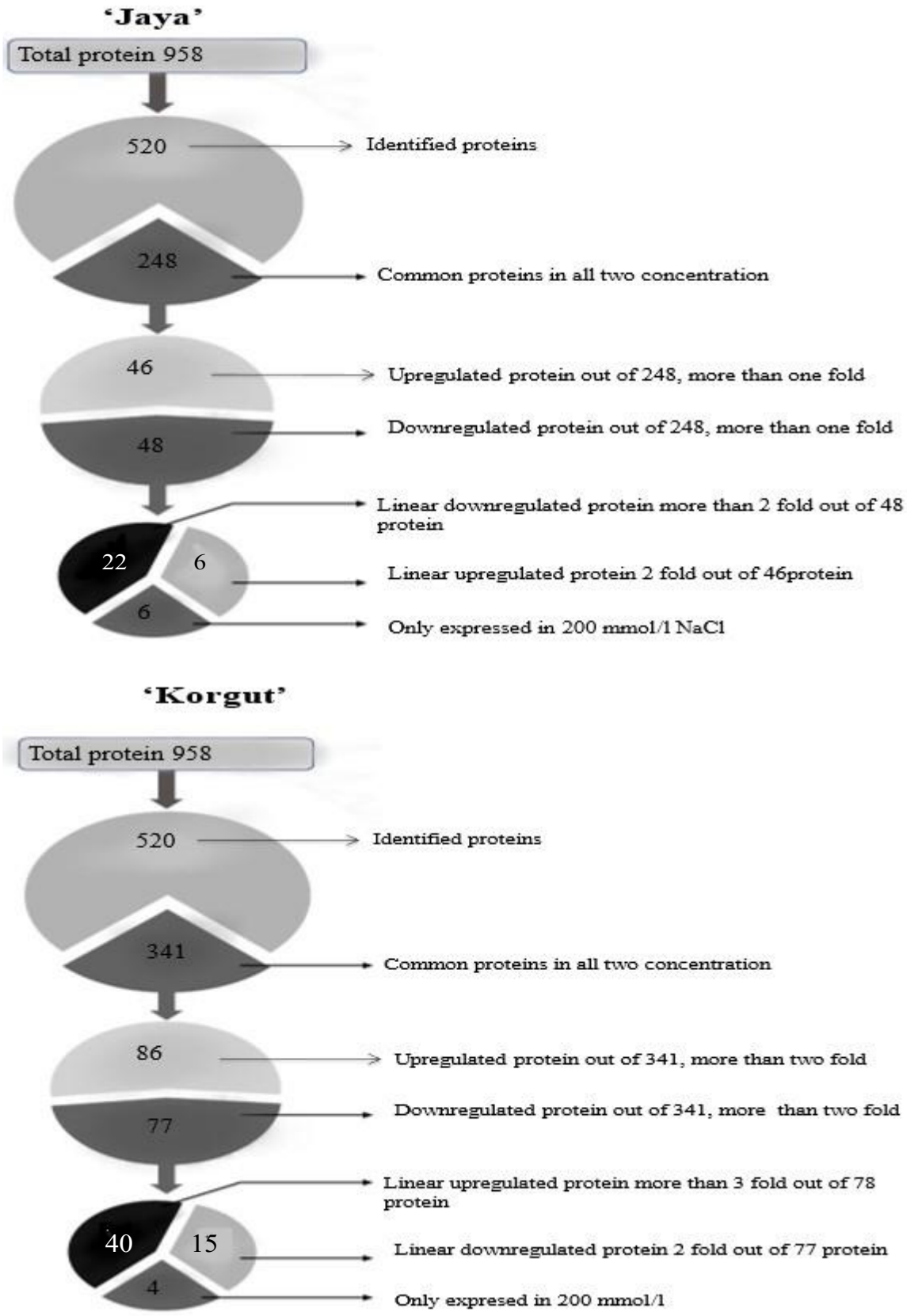


Fig. 3.11.3: Distribution of the total proteins at two different concentrations (control and 200 mmol/l NaCl) in 'Jaya' and 'Korgut' varieties treated for 21 d.

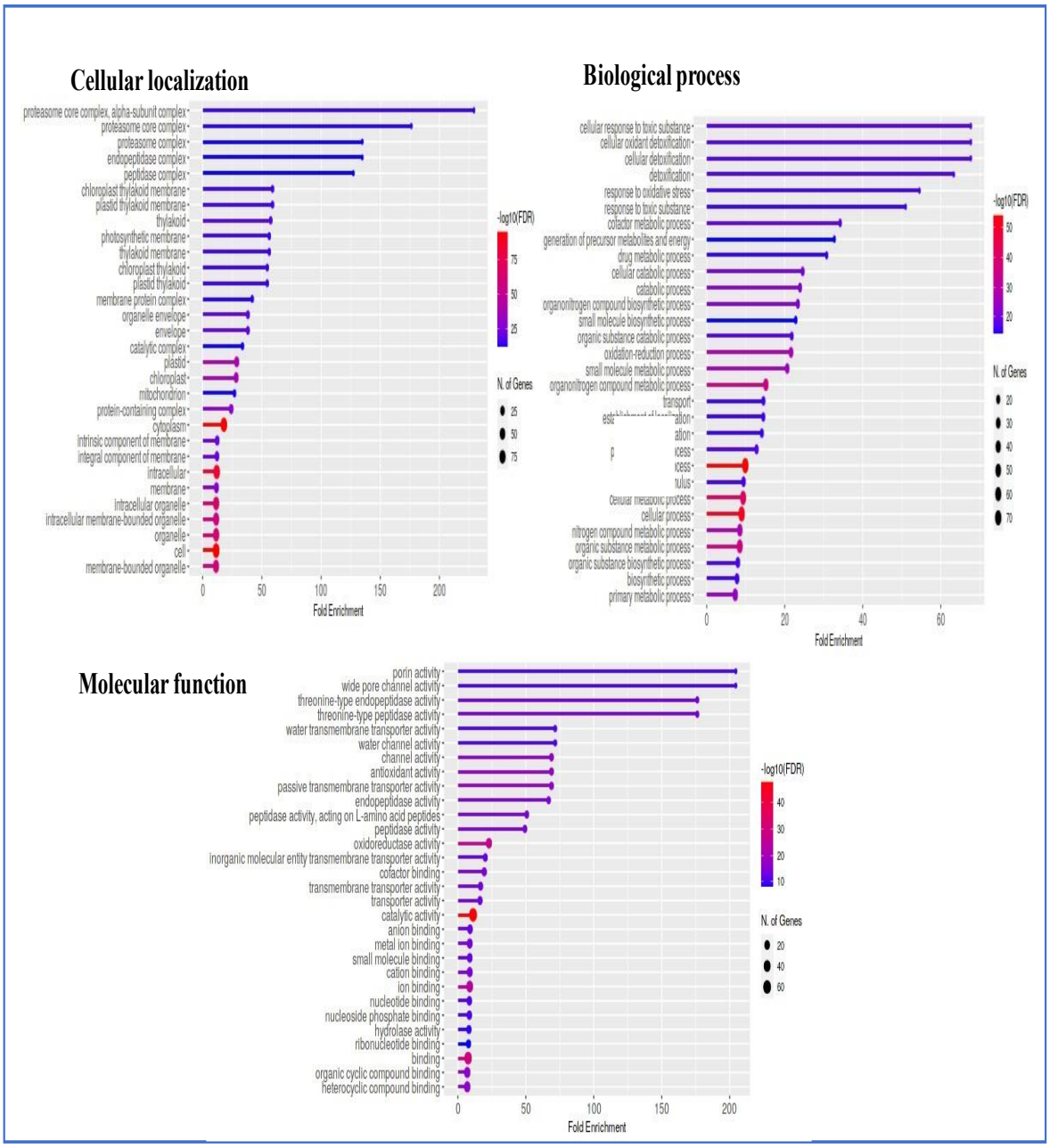


Fig. 3.11.4: Gene functions of 46 upregulated proteins in the 'Jaya' variety treated with NaCl for 21 d. The frequency distribution of identified proteins with their cellular localization biological process and molecular function was characterized based on the *i*Proclass database, and the assignment of functions was based on gene ontology (GO).

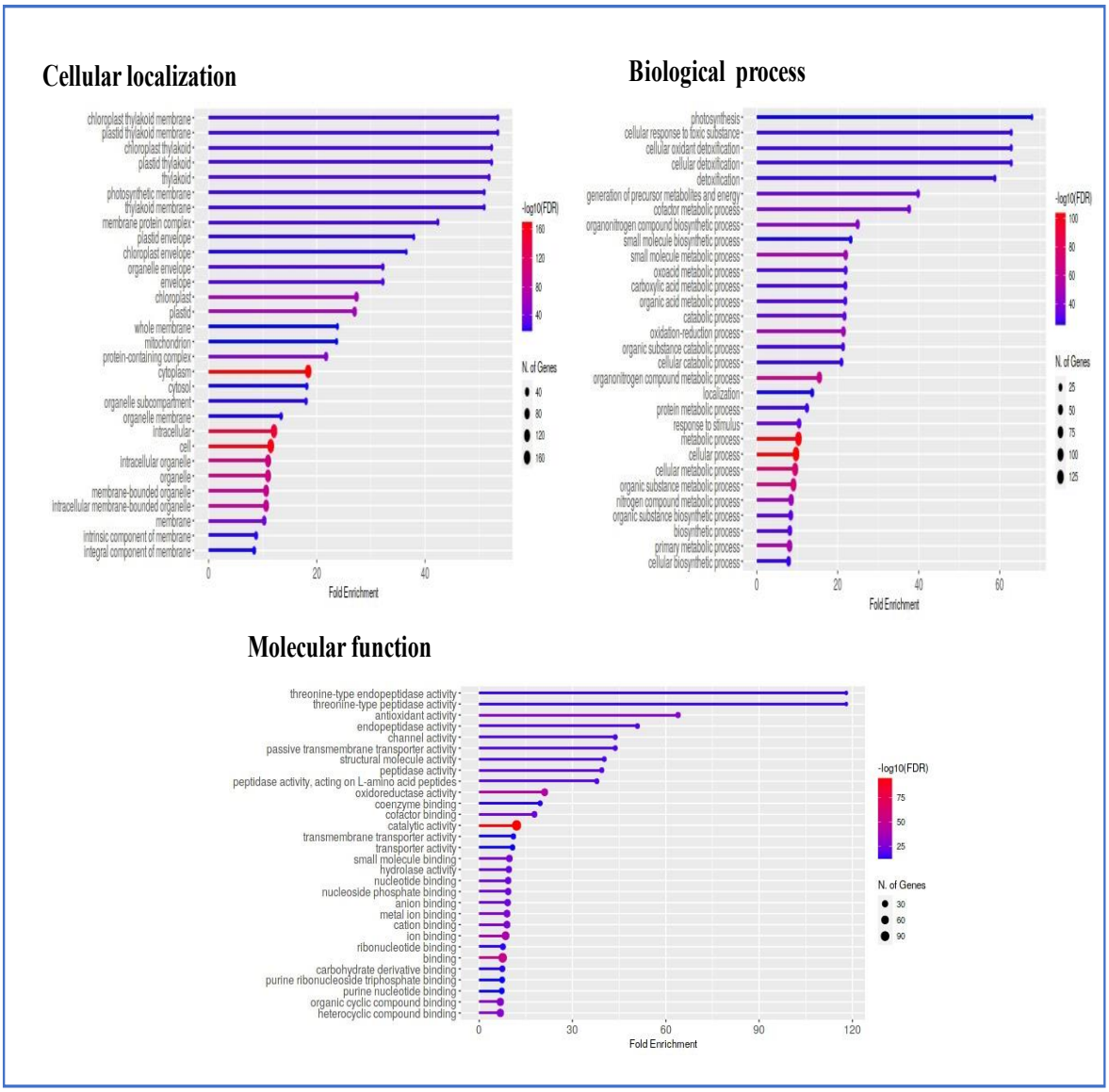


Fig. 3.11.5: Gene functions of 86 significantly upregulated proteins in the 'Korgut' variety treated with NaCl for 21 d. The frequency distribution of identified proteins with their cellular component biological process and molecular function was characterized based on *i*Proclass database, and the assignment of functions was based on gene ontology (GO).

Table 3.11.1: Differentially expressed proteins in 'Jaya' and 'Korgut' varieties treated with 200 mmol/l NaCl for 21 d.

Uniport	Gene Id	description	Function	'Korgut'	Jaya'
Photosynthesis				**log2(FC)**	
A2XU61	Os04g38410	Chlorophyll a-b binding protein CP24, chloroplast precursor,	PS light reaction photosystem	4.61	-3.21
B0FFP0	B0FFP0_ORYSJ	3 kDa polypeptide of photosystem II	photosystem II oxygen-evolving complex	5.23	-4.9
A2YML1	Os07g37240.1	Chlorophyll A-B binding protein,	PS light reaction photosystem	2.32	-2.31
A2ZD01	Os11g13890.1	Chlorophyll a-b binding protein M9, chloroplast precursor,	PS light reaction. photosystem	6.23	-3.34
A2YKQ6	Os07g25430	Photosystem I reaction center subunit IV A, chloroplast precursor,	PSI polypeptide subunits	3.21	-2.31
A2YY54	Os08g44680	Photosystem I reaction center subunit II, chloroplast precursor,	PSI polypeptide subunits	4.92	1
Q0PMC4	Os10g21192	photosystem Q, putative, expressed	PS light reaction	2.691	1
B8A7M8	Os01g49190	ATP synthase beta chain, mitochondrial	PS light reaction ATP synthase	6.18	1
J3R7S9	Os04g16740	ATP synthase alpha chain, putative	PS.lightreaction.ATP synthase	4.68	1.1
A2YLT1	Os07g32880	ATP synthase gamma chain, chloroplast	PS.lightreaction.ATP synthase	4.8	1
D3DF43	Os09g08910	ATP synthase alpha chain, mitochondrial,	PS.lightreaction.ATP synthase	6.03	4.4
B8AKX5	Os03g0786100	Hydroxy acid oxidase 1, putative, expressed	Photosynthesis; photorespiration, pathway	5.72	0.002
A2YCP9	Os12g22030	Serine hydroxymethyltransferase, mitochondrial precursor	One-carbon metabolism; pathway	2.553	0.015
A2ZJQ0	Os12g19470	Ribulose bisphosphate carboxylase small chain C,	PS.calvincyle.rubisco small subunit	4.71	1.01

A2Y650	Os05g41640	Phosphoglycerate kinase, chloroplast precursor	PS.calvincyle.phosphoglycerate kinase	3.717	0
A2XU83	Os04g38600	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplast precursor,	PS Calvin cycle GAP; 4.9	3.78	0.0
B8ACY2	Os01g02880	Fructose-bisphosphate aldolase, chloroplast precursor,	PS calvincyle aldolase	2.436	0.00
B8AEU4	Os02g57180	NADH-ubiquinone oxidoreductase 39 kDa subunit, mitochondrial precursor,	Mitochondrial electron transport	0.782	0
B8BG64	Os10g17280	ATP synthase gamma chain, mitochondrial precursor,	Mitochondrial electron transport	3.545	0.017
A2X7C5	Os02g40830	Succinyl-CoA ligase beta-chain, mitochondrial precursor,	TCA / org.	1.1	4.32
Q6ZFJ3	Os02g01340	Ferredoxin--NADP reductase, leaf isozyme 2, chloroplastic	Regulating the relative amounts of cyclic and non-cyclic electron flow	4.8	2.1
B8B729	Os07g04240	Succinate dehydrogenase flavoprotein subunit, mitochondrial	TCA / org.	2.725	-5.4
Q0D5P8	Os07g36080	Oxygen-evolving enhancer protein 3, chloroplastic, OEE	Electron transport pathway of photosynthesis activity	5.1	-3
Q9C9K2	At1g76560	Calvin cycle protein CP12-3, chloroplastic	Acts as a linker essential in the assembly of a core complex of PRK/GAPDH	3.77	-3.2
A6N154	Os03g39610	Chlorophyll a-b binding protein, chloroplast precursor, putative, expressed	PS light reaction photosystem II.LHC-II	3.658	-4

Carbohydrate and energy metabolism					
Q0J8A4	Os08g0126300	Glyceraldehyde-3-phosphate dehydrogenases, GAPDH	Converting D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate.	5.32	-4.32
Q01859	Os05g0553000	ATP synthase bate subunit, ATPB	Mitochondrial membrane ATP synthase (F_1F_0 ATP synthase or Complex V)	5.21	3.2
Q7XDC8	Os10g0478200	Malate dehydrogenase, mitochondrial	Carbohydrate metabolism process	3.21	3.8
P12085	LOC_Osp1g00410	ATP synthase subunit beta, chloroplastic	Carbohydrate metabolism process	3.24	-2.15
Q07661	Os07g0492000	Nucleoside diphosphate kinase, NDKR	Synthesis of nucleoside triphosphates	3.91	-1.9
Q7G065	Os01g0633100	Glucose-1-phosphate adenylyltransferase 2, cytosolic	Involved in the synthesis of starch	2.21	-3.12
Stress and defense					
Q6Z7B0	Os02g0115900	Heat shock 70 kDa protein 4, putative, expressed, BIP	Seed storage proteins during seed maturation	1.2	1.9
A3C5A7	Os10g0450900	Glycine-rich protein 2, putative, expressed	Responsible for the plasticity of the cell wall	2.4	1.1
A2Z2G1	Os09g30412.1	Heat shock protein 81-3, putative, expressed	Stress, abiotic, heat	3.1	2.1
P48642	Os02g0813500	Glutathione reductase, cytosolic, GRC2	Maintains high levels of reduced glutathione in the cytosol.	3.2	3.4
Q8S3R2	Os02g0707100	Monodehydroascorbate reductase 2, peroxisomal, MDAR2,	Antioxidant against reactive oxygen species (ROS) and nitric oxide (NO).	4.2	1.1
Q0D9C4	Os06g0727200	Catalase isozyme B	Protect cells from the toxic effects of hydrogen peroxide	4.5	0.0
Q0E4K1	Os02g0115700	Catalase isozyme A,	Protect cells from the toxic effects of Hydrogen peroxide	2.1	1.1
Q0DRV6	Os03g0351500	Superoxide dismutase [Cu-Zn] 1	destroys radicals	3.2	1
Q9FR35	Os01g0675100,	Peroxiredoxin-2C	destroys radicals	2.6	0.0

Q948T6	Os08g0191700	Lactoylglutathione lyase, GLYI	Involved in the detoxification of methylglyoxal.	2.12	0.0
P14655	Os04g0659100	Glutamine synthetase, chloroplastic	Responsible for the reassimilation of the ammonia generated by photorespiration	3.5	2.1
Q0DKF0	Os05g0169100	60S ribosomal protein L10-2	Ribosomal protein; protein.synthesis	1.3	0.0
Q0DKF0	Os08g0130500	60S acidic ribosomal protein, putative, expressed	Ribosomal protein; protein.synthesis	1.53	0.0
B8A7P8	Os01g67134.1	60S ribosomal protein L5-1, putative, expressed	Ribosomal protein; protein.synthesis protein	2.6	0.0
P49398	Os02g0105900,	40S ribosomal protein S4, putative, expressed	Ribosomal protein; protein.synthesis	2.4	0.0
A0A0N7KJ13	Os04g0413600	40S ribosomal protein S14, putative, expressed	Ribosomal protein; protein.synthesis	2.7	0.0
A2X6N1	Os02g37862.1	60S ribosomal protein L6, putative, expressed	Ribosomal protein; protein.synthesis	2.691	0.0
A2XDL4	Os03g10340.1	40S ribosomal protein S3a, putative, expressed	Ribosomal protein; protein.synthesis	1.665	0.0
B8AK27	Os03g14530.1	40S ribosomal protein S20, putative, expressed	Ribosomal protein; protein.synthesis	02	0.0
B8ARB0	Os04g42270.2	60S ribosomal protein L23a	Ribosomal protein; protein.synthesis	1.638	0.0
A2XX38	Os04g50990.1	60S ribosomal protein L12, putative, expressed	Ribosomal protein; protein.synthesis	2.217	0.0
B8ATU3	Os04g51630.2	60S ribosomal protein L7-2	Ribosomal protein; protein.synthesis	1.379	0.0
A2Y0K0	Os05g06310.1	60S ribosomal protein L18, putative, expressed	Ribosomal protein; protein.synthesis	1.737	0.0
B8AZ52	Os05g11710.1	60S ribosomal protein L11, putative, expressed	Ribosomal protein; protein.synthesis	2.445	0.0
B8AW24	Os05g19370.1	60S ribosomal protein L15, putative, expressed	Ribosomal protein; protein.synthesis	2.725	0.0
A2Y3W4	Os05g30530.1	40S ribosomal protein S4, putative, expressed	Ribosomal protein; protein.synthesis	2.586	0.0
A4Q8X0	Os01g73880.1	Eukaryotic translation initiation factor 4E-1, putative, expressed	Protein.synthesis.initiation	2.451	0.0
A2XZF9	Os05g01450.1	Eukaryotic translation initiation factor 3 subunit 5	Protein.degradation.ubiquitin.proteasome	2.621	0.0

Ion and Minerals transport					
Q7XSQ9	Os04g0559700	Plasma membrane intrinsic protein 1-2;	Transport of water and small neutral solutes	3.2	0.0
Q6K215	Os02g0629200	Probable aquaporin PIP2-2	Transport of water and small neutral solutes	3.1	0.0
Q6K215	Os02g0629200	Probable aquaporin PIP2;1	Transport of water and small neutral solutes	2.2	0.0
Lipid metabolism and cell wall metabolism proteins					
A2ZHF7	Os12g02340	LTP family protein precursor expressed	Lipid metabolism	5.1	0
A2XNF8	Os04g46910	Actin-depolymerizing factor, putative, expressed	Cell. organization	3.2	1.1

3.12Effect of salt stress on transcriptome analysis

3.12.1 Antioxidant gene expression (SOD and APX)

The results of qRT-PCR gene expression analyses showed greater expression of SOD and APX genes in the salt-tolerant 'Korgut' than in salt-sensitive 'Jaya'. In 'Jaya,' the expression level of the SOD gene increased by 2.9 and 5-fold at 120 and 200 mmol/l of NaCl concentration, respectively, as compared to its control. However, in the 'Korgut' variety, the expression level increased by 7.37 and 51.9-fold at the same salt concentration compared to their respective controls (Fig. 3.12.1 a).

The expression level of APX increased by 1.6 at 120 mmol/l while showing a lower expression level (0.658) at 200 mmol/l of NaCl concentration in 'Jaya' as compared to its control. While in the 'Korgut,' the expression level increased by 6.44 and 16-fold at 120 and 200 mmol/l of NaCl concentration, respectively, compared to its control (Fig. 3.12.1 b).

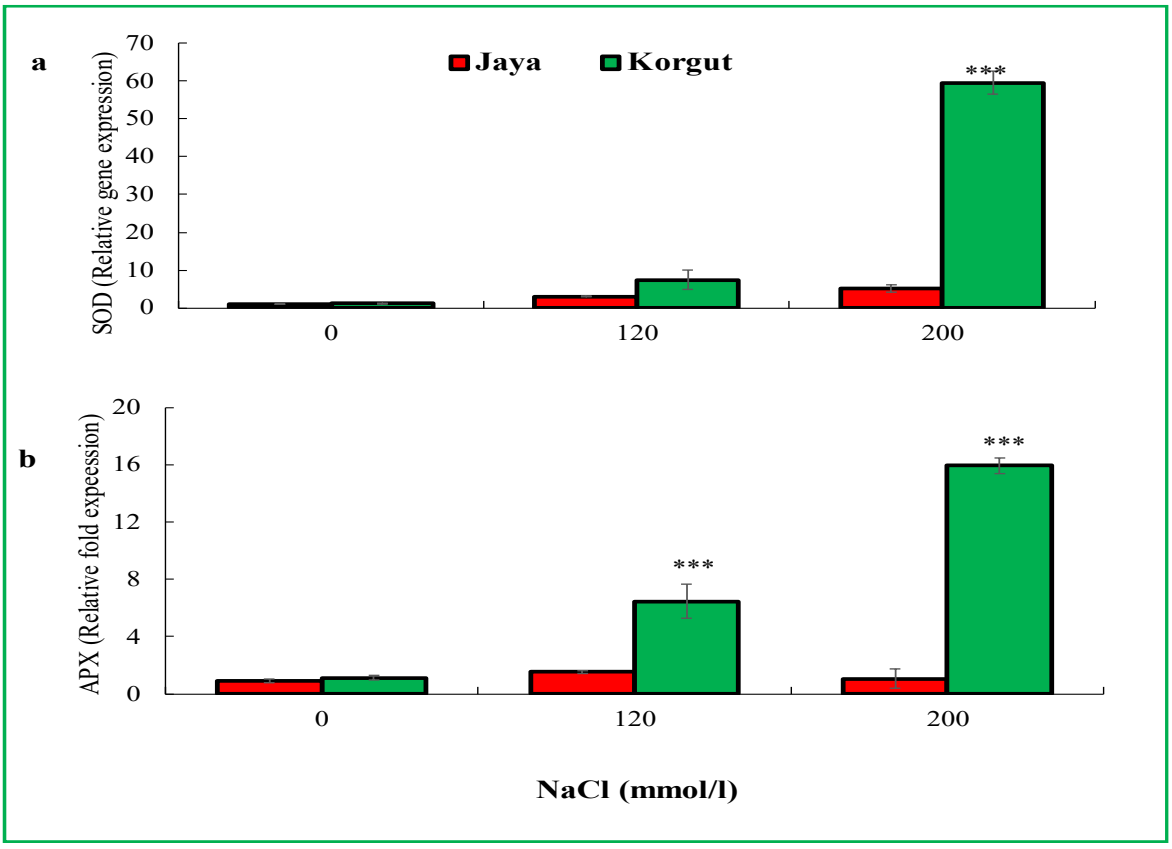


Fig. 3.12.1: Effect of NaCl on gene expression of antioxidant enzymes SOD (a) and APX (b) in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (***) indicates the significance at $p< 0.01$. Standard deviation (±SD) indicates the means of three replicates.

3.12.2 Gene expression of proline synthesis gene (OsP5CS1)

The expression level of the OsP5CS1 gene, related to proline biosynthesis, increased 1.36 and 3.1-fold at 120 and 200 mmol/l of NaCl treatment, respectively, in a 'Jaya' variety as compared to its control. While in 'Korgut,' variety expression of OsP5CS1 increased to 1.39 and 3.96 -fold at the same concentration compared to their control (Fig. 3.12.2).

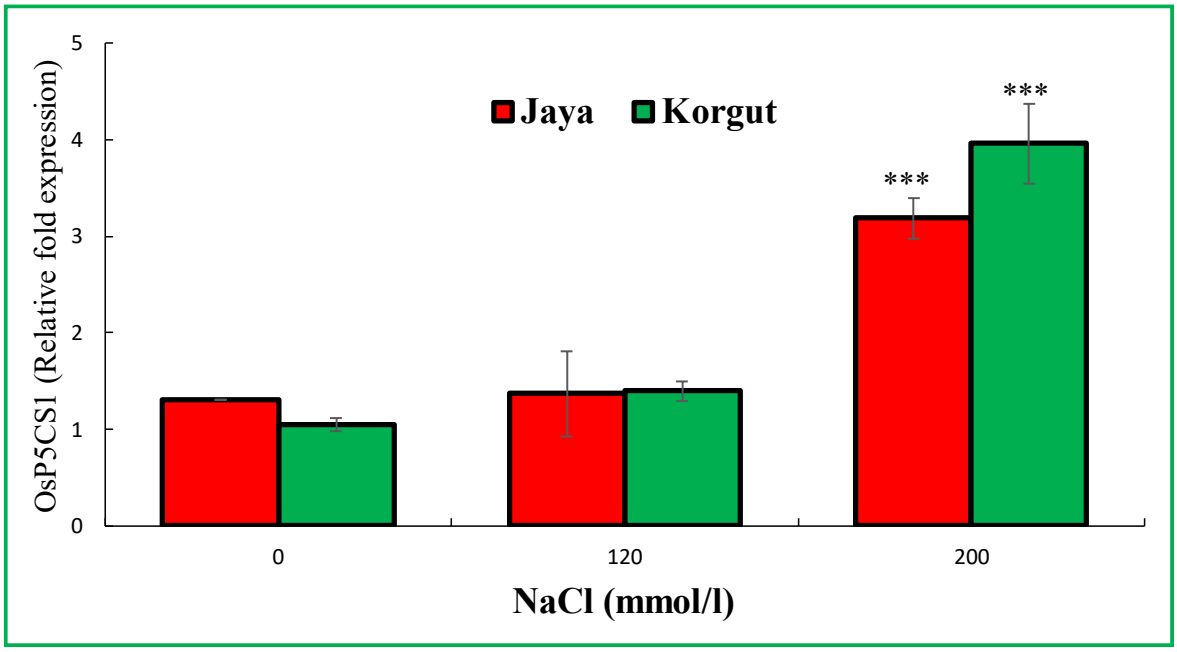


Fig. 3.12.2: Effect of NaCl on proline synthesis gene expression (OsP5CS1) in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (***) indicates the significance at $p< 0.01$. Standard deviation (±SD) indicates the means of three replicates.

3.12.3 Expression of transporter genes (OsHKTs)

The expression of OsHKT1;1 (a Na^+ transporter) gene was upregulated in the salt-sensitive 'Jaya' variety while downregulated in the salt-tolerant 'Korgut' variety under salinity stress. The highest transcript level of OsHKT1;1 gene in 'Jaya' was recorded to be 11.99 and 32-fold at 120 and 200 mmol/l NaCl treatment, respectively, as compared to its control (Fig. 3.12.3 a). In 'Korgut,' however, the expression level of OsHKT1;1 was downregulated by 0.499 and 0.067-fold only for the same concentration of NaCl treatment, respectively, compared to its control (Fig. 3.12.3 a).

In contrast, the expression level of OsHKT2;4 transcripts, a K^+-Na^+ co-transporter, increased in the 'Korgut' variety by 1.5 and 3.65-fold at 120 and 200 mmol/l NaCl treatment, respectively, as compared to its control (Fig. 3.12.3 b).

In 'Jaya,' however, the expression level of OsHKT2;4 was downregulated by 0.981 and 0.341-fold at the same concentration compared to their control (Fig. 3.12.3 b).

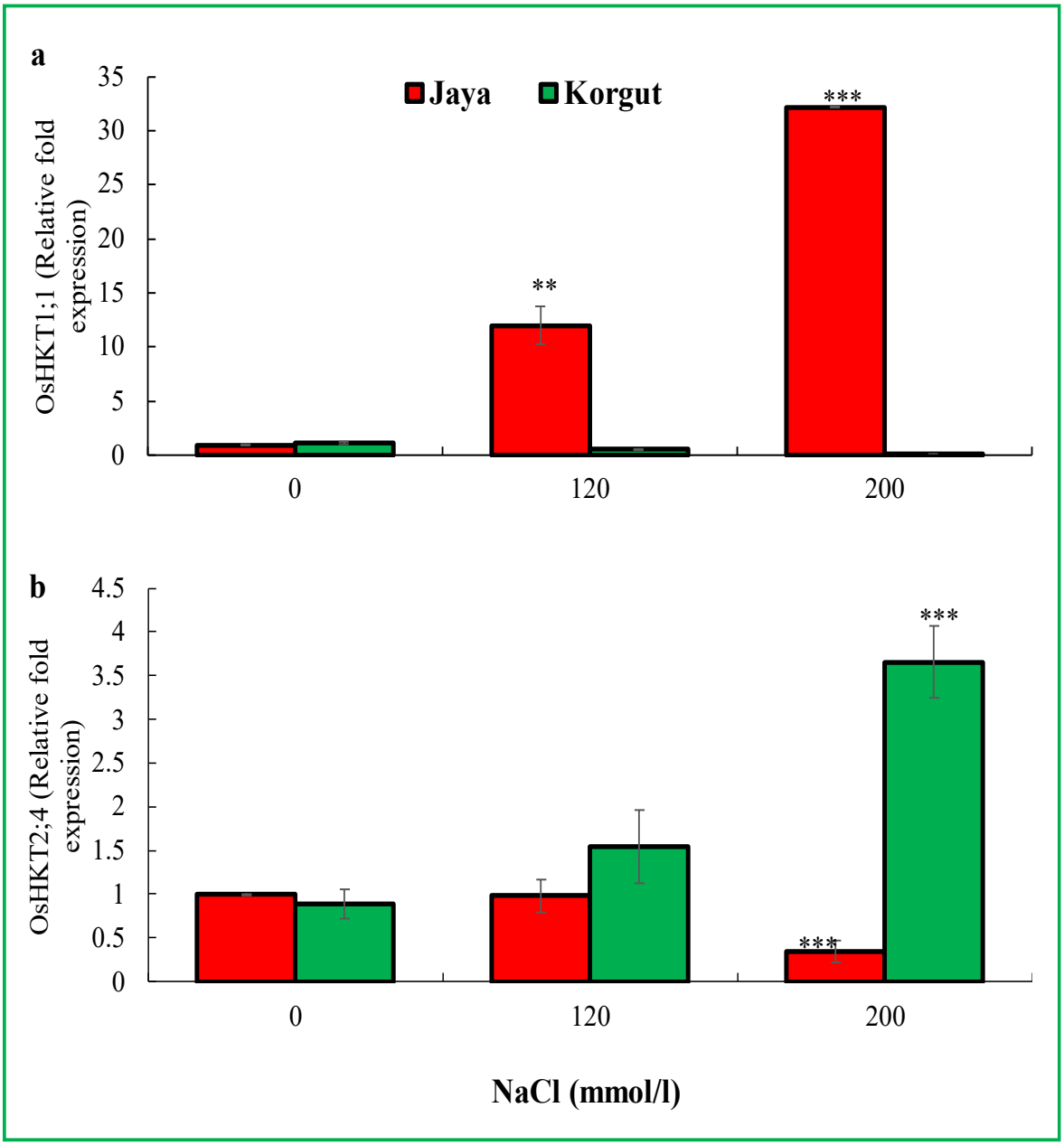


Fig. 3.12.3: Effect of NaCl on gene expression of OsHKT1;1 (a), and OsHKT2;4 (b) in 'Jaya' and 'Korgut' varieties of rice treated for 21d. (**), (***) indicates the significant at p< 0.005, *p*< 0.01 respectively. Standard deviation (±SD) indicates the means of three replicates.

3.13 Principal Component Analysis (PCA)

Thirty-six variables (parameters) of data collected from both salt-sensitive 'Jaya' and salt-tolerant 'Korgut' treated with different concentrations of NaCl were used to assess the salt tolerance capacity of the varieties by the PCA. The PCA demonstrated the different responses of rice genotypes under salt stress conditions. PC1 accounted for 58.51% of the variation and was positively affected by photosynthetic pigments (Car), F_O, qNP, Na^+R, Na^+L, Cl^-L, Cl^-R, H_2O_2, OH^*, MSI, MDA, OL, and the antioxidant enzymes (APX and AsA). PC2 accounted for 16.76% of the variation in the PCA plot and was positively correlated with K^+R, K^+L, SL, RL, FWS, DWR, RWC, gs, qP, Fv/Fm, SOD, CAT, and ALA (Fig. 3.13).

However, 'Korgut' variety was positively correlated with RWC, growth, biomass, K^+ content in leaf and root, photosynthesis, carotenoids, antioxidants like SOD, CAT, APX, AsA ALA, and OA (fatty acid) even at higher levels of NaCl, the distance between control and 200 mmol/l NaCl in 'Korgut' was lesser, indicating that it thrived well under increased salinity levels than the distance between different concentrations of NaCl in 'Jaya' showing difference in its response at higher NaCl concentrations. Higher salt concentrations in 'Jaya' was closely related to ion accumulation seen as increased Na^+R, Na^+L leading to oxidative damage including ROS generation H_2O_2 and $OH\bullet$ increase in MDA, MSI affecting Fo and qNP and others parameter (Fig. 3.13).

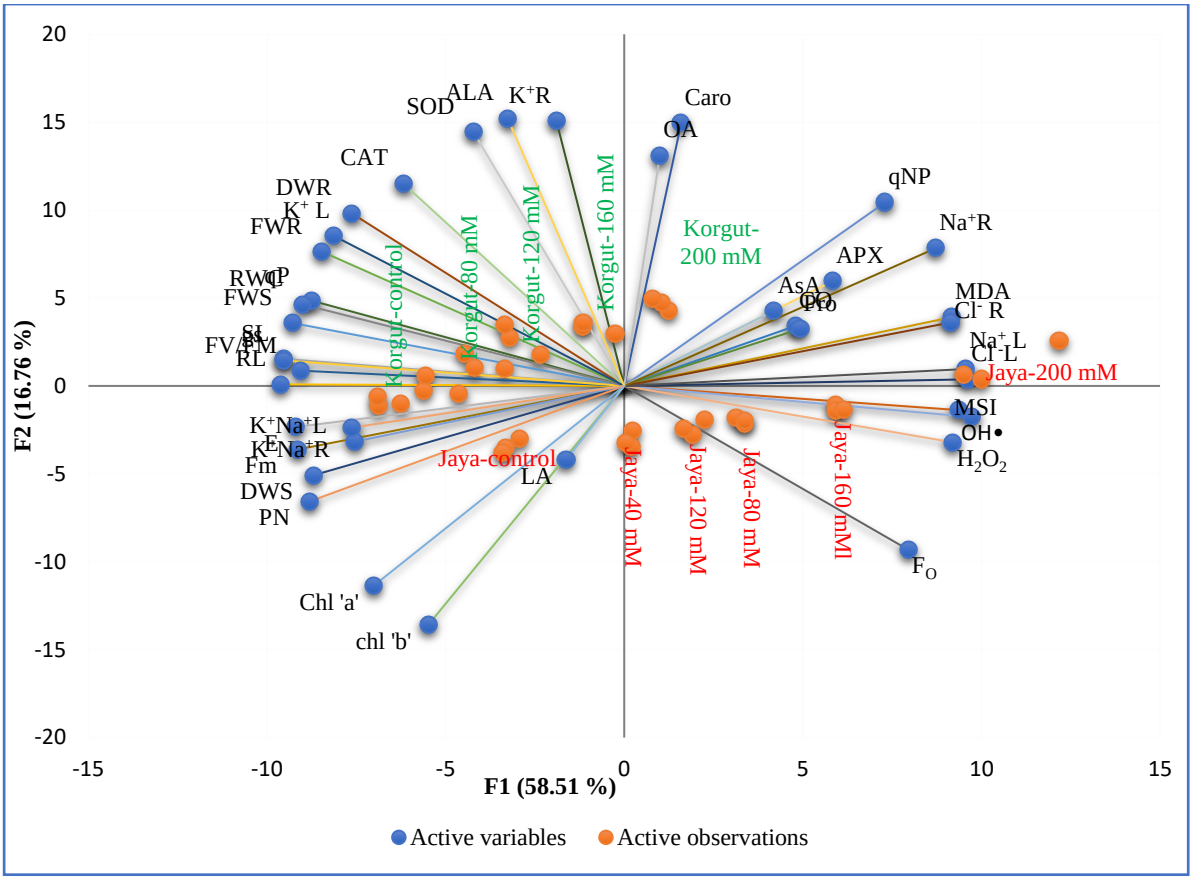


Fig. 3.13: The scatter plot of the F1/F2 plane shows the relationships between the studied rice plants and all the measured characteristics. Considered variables including: leaf relative water content (RWC), membrane stability index (MSI), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), corbonyl conyent (CO) proline (Pro), chlorophyll a (Chl 'a'), chlorophyll b (Chl 'b'), carotenoids (Caro), ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), leaves K^+ concentration (K^+L), roots K^+ concentration (K^+R), leaves Na^+ concentration (Na^+L), roots Na^+ concentration (Na^+R), leaves K^+/Na^+ ratio (K^+Na^+L), roots K^+/Na^+ rate (K^+Na^+R), leaves chloride concentration (Cl^-L), roots chloride concentration (Cl^-R), quantum efficiency of PSII system (Fv/Fm ratio), maximal flurosence (Fm), photochemical quenching (qP), non- photochemical quenching (qNP), Net photosynthesis rate (P_N), transpiration rate (E), stomatal conductance (g_s), alpha-Linolenic acid (ala), linoleic acid (LA) and oleic acid (OA), fresh weight of shoot (FWS) fresh weight of root (FWR), dry weight of shoot (DWS), dry weight of root (DWR), shoot length (SL) and root length (RL).

CHAPTER 4

DISCUSSION

“Discussion and argument are essential parts of science; the most incredible talent is the ability to strip a theory until the simple basic idea emerges with clarity.”

Albert Einstein

Salt stress in the coastal saline soil of Goa is a severe detriment limiting the productivity of *Oryza sativa*. Keeping in mind the damaging effects of salinity on crop productivity, we designed the present study to understand the physiological marker better and elucidate the mechanism of salt tolerance by comparing morphological, physiological, biochemical, and molecular characteristics in salt-sensitive and tolerant rice varieties. In this study, the uptake of Na^+ and Cl^- in leaf and root tissue was analyzed, and its subsequent effect on the external and internal morphology of the leaf is discussed. The present study also incorporates the sodium and potassium transporter gene expression data. Other physiological parameters, such as relative water content, photosynthesis, and pigment analysis, are also discussed. This study also provides supporting evidence for the involvement of Na^+ and Cl^- in the oxyradical formation in the form of OH• and H_2O_2 levels, which was associated with the oxidative damage caused to the lipids and proteins. To counteract the toxic effect of salt stress in the form of ROS generated, rice plants employed antioxidative responses as a defense mechanism by increasing the activity and expression of SOD, CAT, and APX. This is further confirmed by a fatty acid composition that showed biosynthesis of unsaturated fatty acids such as α-linolenic acid (18:3ω3) and oleic acid (18:1ω9), which contribute to the maintenance of membrane integrity and indicate adaptive strategy for protection against the oxidative damage. In addition, a comparative proteomic study of tolerant and sensitive rice varieties in response to salt stress is also discussed.

4.1 Relative water content (RWC)

Observed higher leaf RWC in salt-tolerant 'Korgut' variety to the extent of 49-72% compared to the salt-sensitive 'Jaya' variety in our study (Fig. 3.2) suggest a better water availability to maintain cell turgidity and normal metabolic process under salt stress in 'Korgut' variety. RWC, an indicator of plant water status and early response to salt stress, affects plant water status by building osmotic solute in the soil, preventing water uptake by roots and further transportation to shoot, thus causing physiological drought-like conditions. Uptake of Na^+, to an extent, may be desirable as a way to build an osmotic potential to facilitate water absorption and sustain turgor, but uptake of excess Na^+ ion becomes toxic as observed in our study, more so with salt-sensitive variety than in salt-tolerant variety. Morphological changes observed in our study (Fig. 3.3.1; A and B) may also play a role in maintaining better plant water relations by preventing water loss in salt-tolerant 'Korgut' than in salt-sensitive 'Jaya'. The same is discussed in more detail

in section 4.4. Our data is in concert with work carried out by other groups on salt stress. Zhao et al. (2014) reported a higher RWC in the salt-tolerant rice variety (FL478) than in salt-sensitive (IR64), indicating the role of RWC in maintaining cell turgidity and normal metabolism in the tolerant variety under stress conditions. Rahneshan et al. (2018) reported a reduction in the RWC in the salt-sensitive *Pistachio* variety compared to the salt-tolerant variety at 100 mM NaCl concentration. Likewise, Sarabi et al. (2017) also showed that in the leaves of the salt-tolerant melon variety, RWC was not affected at low NaCl concentration (30 mM), while it slightly reduced at 60 mM and 90 mM salinity as compared to salt-sensitive variety, and confirmed that decreasing the cellular osmotic potential (higher RWC) allowed the roots to the uptake of sufficient amount of water to maintain cell turgidity and potentially to improve their hydration status in comparison to sensitive variety. Our results with RWC are further supported by Win and Oo (2015), working with different varieties of cowpea and wheat, respectively, grown with NaCl and suggested that the genotypes maintained their tissue water content under salinity stress by keeping higher RWC and, thereby, higher salt tolerance. Stępien and Kłbus (2006) also showed in *Cucumis sativa* that water status is susceptible to salinity and is dominant in determining the plant's response to salt. Odjegba and Chukwunwike (2012) in *Amaranthus hybridus* and Menezes et al. (2017) in *Amaranthus cruentus* reported a similar reduction of RWC under different salt concentrations, suggesting excess Na^+ stress reduces the turgor pressure and soil water availability thereby inhibiting the uptake of water, resulting into decreased RWC which was suggested to lower the productivity. Mahlooji et al. (2018) have also observed that the salt-tolerant barley genotype (Khatam) maintained its RWC, while the sensitive genotype (Morocco) showed a significant reduction in RWC at high salinity and showed that impaired water absorption and high Na^+ accumulation was a result of reduced RWC in salt-sensitive variety.

4.2 Sodium, Potassium, and Chloride content

In our study, we observed a greater accumulation of Na^+ in the root (25-fold) and leaves (15-fold) of the salt-sensitive 'Jaya' variety than seen in the salt-tolerant 'Korgut' variety (Table 3.4.1). Observed lower RWC content (Fig. 3.2) in the 'Jaya' variety in spite of higher salt concentration suggested a poor role of Na^+ in building osmotic potential. As RWC value was much lesser in 'Jaya' than in 'Korgut,' suggesting higher Na^+ toxicity in the 'Jaya' variety. An increase in Na^+ content is always suggested to be accompanied by Cl^- an accumulation (Tavakkoli et al., 2010) and K^+ loss (Wu et al.,

2015). Uptake of high Cl^- ions are also toxic and impair photosynthesis and growth in salt-sensitive 'Jaya'. On the other hand, the salt-tolerant 'Korgut' variety had less accumulation of Cl^- ions in the root and shoot (Table 3.4.1), suggesting superior root selectivity to Cl^- uptake. Higher level Cl^- ion in 'Jaya' may also indicate a homeostatic imbalance in ion uptake (Cl^-, Na^+, and K^+) that leads to the failure to regulate the apoplastic and symplastic activities and cell dehydration in salt-sensitive variety. In addition, the observed lower level of K^+ in 'Jaya' (Table 3.4.1) may also interfere with insufficient stomatal regulation (Fig. 3.5.1 c), resulting in increased water loss and decreased photosynthesis which directly affects plant growth, as seen was in our study (Fig. 3.1). Observed higher accumulation of K^+ in roots and leaves of 'Korgut' than the 'Jaya' variety (Table 3.4.1) indicates that K^+ may also be involved in inorganic osmoticum, helping in maintaining higher RWC (Fig. 3.2). This higher K^+/Na^+ ratio in 'Korgut' (Table 3.4.1) is also positively correlated with CO_2 assimilation (Fig. 3.5.1 a), suggesting higher water efficiency of photosynthesis in 'Korgut' (Fig. 3.5.1 e). Na^+ as a cation competes with the same transporter system as K^+, and decreased K^+ status in 'Jaya' indicates that Na^+ not only interference with K^+ translocation from soil to root to shoot by competing with K^+ resulting in lower K^+ uptake causing higher accumulation of Na^+ and its toxic effects, and poorer growth. These results are further discussed in section 4.11 with expression studies of OsHKT1;1 and OsHKT2;4, transporter genes for Na^+ and K^+.

Similar observations regarding the accumulation of Na^+, Cl^-, and K^+ ion has also been reported by other. De Azevedo Neto et al. (2006) observed that the salt-resistant maize variety (BR5033) had less accumulation of Na^+ as compared to the salt-sensitive variety (BR5011), indicating that the tolerant variety was able to exclude sodium more efficiently from leaf cell cytoplasm than sensitive variety. They have also reported a higher reduction in root K^+ content in the salt-sensitive variety than tolerant variety, suggesting that salt-induced shoot growth inhibition in the sensitive variety is mainly due to metabolic changes resulting from ionic imbalance or ion toxicity occurring in the root system. Likewise, Akrami and Arzani (2018) have found that salt-tolerant melon cultivars (Sabouni and Shahabadi-1) had low leaf Na^+ and high leaf K^+ concentrations and enhanced K^+/Na^+ ratio under stress conditions, which resulted in higher fruit yield, while higher Na^+ accumulation in salt-sensitive cultivars (Gargar-2, Gargar-1), suggests higher accumulation of toxic ion (Na^+) demolishes cell enzyme activities, membrane integrity and photosynthetic apparatus under saline conditions. Similarly, Tao et al. (2021) have

reported the relationship between the root's ability to retain K^+ and maintain the leaf K^+/Na^+ ratio, which was a vital determinant of the salinity tolerance mechanism in wheat grown under salinity stress. Chakraborty et al. (2012) demonstrated that reduced tissue Na^+ uptake coupled with a lower Na^+/K^+ ratio in both roots and shoot tissues had been reported in tolerant cultivars of *Brassica juncea* as compared to sensitive cultivars *B. oleracea* and *B. juncea* and concluded that higher Na^+ content in susceptible variety was the major contributor to cell osmolality in shortage of K^+. A strong correlation between the ability to retain tissue K^+ and salt tolerance has been reported for many plant species (Cuin et al., 2012; Wu et al., 2015). Shakri et al. (2022) in rice observed that a higher accumulation of Na^+ caused a K^+/Na^+ imbalance, which disrupts many physiological functions, including germination in salt-sensitive than salt-tolerant, suggesting that roots of salt-tolerant variety may be able to restrict the uptake of Na^+ and Cl^- ions into the plant transport system via root tissues to prevent toxic sodium accumulation hence prevented the inhibitory effects of salt on root growth. Similar results were described in wheat (Iqra et al., 2020), canola (Naveed et al., 2020), maize (Azizian & Sepaskhah, 2020), tomato (Kamanga et al., 2020), rice (Qin & Huang, 2020), and barley (Zeeshan et al., 2020).

Our study also reported a higher accumulation of Cl^- in 'Jaya' over 'Korgut' (Table 3.4.1). Tavakkoli et al., 2010 in beans and Tavakkoli et al. (2011) in barley using salt of calcium, magnesium, and potassium chloride reported that the effect of Na^+ and Cl^- ion may differ. They observed high Cl^- concentrations in both cytoplasm/chloroplast and in the cytoplasm/vacuole and suggested that high Cl^- ion reduced the photosynthetic capacity and quantum yield due to chlorophyll degradation, which may affect the structural integrity of PS II, while high Na^+ interfered with K^+ and Ca^{2+} nutrition and disturbed efficient stomatal regulation which may result in depression of photosynthesis and growth. Hussain et al. (2012) also observed that *Cleopatra mandarin* and *Australian sour* orange are the second most tolerant citrus rootstock with low Cl^- accumulation correlated with high CO_2 assimilation. Geilfus (2018) has described the antagonistic correlation between Cl^- and NO_3^-, which resulted in a reduction in the uptake and storage of nitrogen, an essential source for protein synthesis and many metabolism products. A study by Gao et al. (2014) showed higher concentrations of Na^+ and Cl^- accumulated in leaf apoplast, leading to water loss of cells, plasmolysis, and decreased intercellular spaces in the leaves of potato plantlets grown under salt stress. Wang et al. (2020) reported that Cl^- salinity inhibited the electron flow rate and thus resulted in low ATP synthesis in

tobacco plants, leading to ROS generation and oxidative damage. Azarin et al. (2016) reported that under NaCl stress, there were higher accumulations of Na^+, Na^+/K^+ ratio, and Cl^- in shoot and root while it reduced the K^+, NO_3^- contents in the vegetative stage of rice seedlings, resulting in chlorophyll degradation and hence reduced the photosynthetic capacity.

4.3 Plant growth and biomass

Our results showed that the 'Korgut' variety performed better than the 'Jaya,' showing higher shoot length, root length, and biomass (Table 3.1). The observed decrease in biomass in the salt-sensitive variety may be a result of several reasons, but largely due to the reduction in the root system's water absorption capacity and increased water loss from leaves. The same is suggested on the basis of our data with regard to the root/shoot ratio in the salt-sensitive 'Jaya' and salt-tolerant 'Korgut' variety. The root/shoot ratio under salt stress provides an important clue to the plant's response to salt stress. Our data suggest that shoot and root length are affected by salt stress to a greater extent in the salt-sensitive 'Jaya' variety and showed that 'Korgut' maintained a better root growth than 'Jaya' in response to salt stress, indicating salt tolerance owing to the root system's ability to uptake water and nutrients, maintaining turgor and allowing the ion dilution to prevent ion toxicity. An increased root/shoot ratio can also improve the source-sink ratio for nutrients under the saline condition, leading to better growth.

Salt treatment build-up ion toxicity, as seen in our study (Table 3.4.1), causes nutritional imbalance, interruption the membrane, impairs the ability to detoxify ROS through antioxidant enzymes, and decreases photosynthetic activity, which is directly related to plant growth (Djanaguiraman & Prasad, 2013). Salt-sensitive 'Jaya' showed lesser RWC, higher Na^+ and Cl^- accumulation, and a lower K^+/Na^+ ratio which led to a more significant effect on these parameters, causing reduced growth and biomass compared to the salt-tolerant 'Korgut' variety. Also, the inhibitory effect of higher accumulation of Na^+ and Cl^- in 'Jaya,' resulting in lower growth, may also be due to the diversion of energy from growth and biomass development to exclude Na^+ uptake and compartmentalization to reduce the ion toxicity effects. Diversion of energy may also be required to synthesize compatible solutes to maintain cell turgor under hyperosmotic saline conditions. Our results with more significant proline accumulation in the 'Jaya' variety (Fig. 3.8.5) also indicate the massive diversion of energy to help maintaining the plant water status, probably at the cost of growth.

Others have also observed similar decreased shoot, root length, and biomass results due to NaCl. Chang et al. (2019) also observed that under NaCl stress conditions, there was a reduction in shoot and root growth of the salt-sensitive *Nipponbare* rice variety to a higher accumulation of Na^+ and Cl^-. Likewise, Jamil et al. (2007), with sugar beet and cabbage, observed decreased shoot lengths, root lengths, and dry weights with increasing salt stress levels, suggesting higher Na^+ and lower K^+ accumulations may have caused unbalanced nutrient uptake and reduced water uptake resulting in reduced growth.

Nandhini and Somasundaram (2022) observed a reduction of cell division in the root apex in salt-grown maize resulting in reduced root dry weight. Bañon et al. (2006) observed that salt stress resulted in reduced cell elongation, cell turgor, cell volume, and cell growth in salt-grown *Nerium oleander*. Hasanuzzaman et al. (2013) have suggested that reductions in growth parameters are due to high osmotic stress and ion toxicity. Widodo et al. (2009) showed that salt-sensitive (Clipper) barley grown at 100 mM NaCl for three weeks showed growth reduction of dry weight compared with the tolerant (Sahara) variety, indicating Sahara displayed tolerance to high internal salt tolerance concentration without apparent cell damage and maintained a higher tissue K^+/Na^+ ratio in the cytoplasm through compartmentation of Na^+ into vacuole or by increasing the metabolite levels to cope with the increased osmotic potential, associated with higher plant growth. Djanaguiraman et al. (2003), observed that the rice 'Pokkali' variety was more tolerant to salt stress than 'IR 50', a salt-sensitive variety. Sarker and Oba (2020), in a study with *Amaranthus tricolor* (VA3), a salt-sensitive variety, showed a higher reduction in biomass and a decrease in leaf area and numbers as compared to the (VA 14), a salt-tolerant variety. Menezes et al. (2017) have also shown a decrease in leaf, stem, and root dry mass, total dry mass, and leaf area in *Amaranthus cruentus* and suggested the same due to the creation of osmotic stress that inhibits transport and absorption of water. They also confirmed that inhibition led to hormones-induced sequential reactions that reduced the opening of stomata, assimilation of CO_2, and the net photosynthetic rate. The reductions in biomass due to salinity stress have been investigated by many scientists in several crops such as tomato (Mozafariyan et al., 2013), basil (Mostafa, 2012), and quinoa (Bakhoum & Sadak, 2016; Elsawy et al., 2018), attributed these reductions due to the metabolic disorders causing excess ROS under salt stress.

4.4 Morphological changes in leaves

The observed results with regard to growth and biomass in salt-sensitive and salt-tolerant varieties are also supported by our data with morphological observation (Fig. 3.3.1A, B). Our data suggest that the 'Korgut' variety was more adapted to salt stress due to a decrease in the size of stomata and an increase in the size and number of trichomes. These morphological adaptations led to better plant water status and a tolerant variety. The increase in the size of the trichome may be an adaptational response related to an accumulation of additional salt away from the functional part of the cell (Hameed et al., 2010). The sensitive variety had poor morphological changes and adaptation, leading to greater water loss and loss of productivity. Decreasing the size of stomata (Fig. 3.3.1B; g-i) is a critical, adapted tool to minimize water loss, avoid the physiological drought-like condition, and maintain the metabolic process (Bertolino et al., 2019). The increased number and size of trichomes (Fig. 3.3.1B; a-f) have also increased the thickness of the boundary layer, consequently imposing a physical resistance to gas diffusion, which can reduce the water loss rate more than the CO_2 assimilation rate, resulting in a higher CO_2 assimilation rate (Fig. 3.5.1 a). Densely covered cuticular papillae, a specialized cell structure composed of lignin, on the adaxial and abaxial surface of 'Korgut' leaves compared to 'Jaya' may also play a significant role in better plant water status in 'Korgut'. Thus, better RWC may lead to increased water use efficiency, as was seen with 'Korgut' in our study (Fig. 3.5.1 e).

Several studies have correlated morphological adaptation to salt tolerance. Çelik et al. (2018) reported that salt-tolerant (S04-05) soybean showed higher trichome densities on both surfaces of the leaves compared to the salt-sensitive (Ataem-7) variety, suggesting trichomes under extreme saline conditions excretes the excess salt and play an adaptive role which bettered plant water status in relation to oxidative metabolism. Sletvold et al. (2010) also reported similar results with *Arabidopsis lyrata* (Brassicaceae) under drought and salinity conditions, suggesting that increased trichome density is an inducible defense mechanism against herbivores and water deficiencies for plants. Benz and Martin (2006) examined the relationships between water and carbon dioxide gas exchange parameters and leaf trichome cover in 12 species of *Tillandsia* that exhibited a wide range in trichome size and trichome cover and found that trichome-enhanced boundary layer had negligible effects on *Tillandsia* gas exchange and concluded that they did not significantly reduce transpirational water loss. Hegebarth et al. (2016) and Busta et al. (2017) in *Arabidopsis thaliana* have observed that the presence of trichomes under salinity increases the

thickness of the epidermis, and the content of long-chain fatty acids significantly than that in other epidermal cells, which was suggested to be helpful to reduced evaporation and better regulation of leaves temperature. Dolatabadian et al. (2011) observed an increased number of trichomes from epidermal cells in salt-grown soybean and suggested their role in increased tolerance to salt stress. These results also support those previously documented by Kemp and Cunningham (1981) that dense trichomes can modulate leaf heat balance and photon interception and consequently affect CO_2 assimilation in melon (Kaya et al., 2007), basil (Barbieri et al., 2012), salt marsh species (Maricle et al., 2009), and tomato (Sánchez-Rodríguez et al., 2010). The occurrence of dense trichome layers, even in tropical species, has also been related to protection against drought and high-light-intensity conditions (Ichie et al., 2016). Likewise, Melo et al. (2021) observed increased trichome density and subsequently decreased stomatal pore size in salt-grown *Calotropis procera*. Similar observations of increasing stomatal density and reduced size in barley plants were positively correlated with salt tolerance by Zhu et al. (2015). Hasanuzzaman et al. (2018), Murphy et al. (2018), and Richardson et al. (2017) showed that a decrease in the number and size of stomata provided a moderate absorption of CO_2 closure to minimize water loss. Waqas et al. (2017) in quinoa, Naz et al. (2010) in pan dropseed, Adolf and Shabala (2013) in Chenopodium, and Mohamed et al. (2020) in rapeseeds have determined that salt tolerance was related to the lower stomatal density and decreased stomatal area. The literature suggested the density and size of stomata and trichomes as reliable salt tolerance indicators.

4.5 Photosynthesis

Our data showed a greater decline in CO_2 assimilation (P_N) with low stomatal conductance (*gs*) and transpiration rate (*E*) in the salt-sensitive 'Jaya' variety than seen in the salt-tolerant 'Korgut' variety (Fig. 3.5.1 a-c), Interestingly, the internal CO_2 (*Ci*) was not significantly affected by the salinity in both varieties (Fig. 3.5.1d), indicating that the reduction of CO_2 assimilation was a result of a combination of both stomatal and non-stomatal factors. Observed higher decline in RWC (Fig. 3.2), greater Na^+ accumulation (Table 3.4.1), and morphological changes in size and numbers of trichomes and stomata size (Fig. 3.3.1A; a-i) in salt-sensitive 'Jaya' variety is also commiserated with a lower net photosynthetic rate in 'Jaya' contrary to salt-tolerant 'Korgut' as all these parameters helped in its water use efficiency (Fig. 3.5.1e). Our result with P_N /*Ci* (Fig. 3.5.1 f) indirectly suggest higher Rubisco activity in 'Korgut' specially at higher salt stress as P_N

/*Ci* an indirect indicator of the carboxylation efficiency, was significantly higher in 'Korgut' than 'Jaya' beyond 80 mmol/l NaCl. Our results with less membrane leakage in the 'Korgut' variety (Fig. 3.7.2) also support better net photosynthesis. Another possible reason for maintaining higher net CO_2 fixation in 'Korgut' may be related to higher K^+ in leaves and root tissue which is an essential element required for the maintenance turgidity and was mainly maintained by carbohydrate translocation; these results were further supported by photosynthesis proteins that were upregulated in 'Korgut' (Table 3.11.1).'Korgut,' being a salt-tolerant variety, maintained better structural integrity and orderliness of chloroplast (Fig. 3.3.2B; a-f), thus better photosynthetic machinery, which is necessary for the conversion of light energy during photosynthesis. The toxic levels of Na^+ and Cl^- ions may detrimentally affect the integrity of the cell and may also affect the activities of RuBP carboxylase and nitrate reductase activity (Tavakkoli et al., 2010). Our data with the chlorophyll fluorescence measurements also suggest the organizational and structural changes in a pigment-protein complex in both the light-harvesting complex and reaction center (Fig. 3.5.2).

Researchers have reported suppression of net photosynthesis and transpiration under salinity. Tavakkoli et al. (2011) noted that the salt-tolerant barley variety (Barque73) had significantly greater photosynthetic rate and water-use efficiency while salt-sensitive (Sahara) reduced photosynthesis mainly by reducing stomatal conductance, suggesting decrease in photosynthesis was due to stomatal factors as stressed plants reduced required K^+ concentrations in an epidermal cell which was responsible for the regulation of stomata opening and closure. Likewise, Qi et al. (2019) also reported a decrease in P_N, gs, and an increase in Ci under K^+ deficiency stress in K-sensitive (D937 and 835) than in K-tolerant (90-21-3 and 099) maize plants. They suggested that K-sensitive maize plants developed fuzzy membranes of thylakoids, which resulted in impaired photosynthetic process and reduced chlorophyll content and chlorophyll fluorescence parameters, which are mainly responsible for the degradation of leaf photosynthetic capacity. Sui et al. (2015) and Yang et al. (2019) have also reported a significant decrease in stomatal conductance, net photosynthetic rate, PSII photochemical efficiency, and intercellular CO_2 concentration (Ci) in salt-sensitive sweet sorghum varieties in contrast to tolerant variety, indicating that the salt-tolerant sweet sorghum was responsible for maintaining high photosynthetic efficiency by contributions of several pathways, such as keeping the stability of the photosynthetic system and enhancing the efficiency of CO_2 fixation. Similarly, Moradi and Ismail (2007) demonstrated a remarkable decrease

in photosynthetic CO_2 fixation, transpiration, and stomatal conductance (*gs*) in salt-sensitive rice cultivars (IR29) as compared to the salt-tolerant cultivar (IR651) after 312 h of exposure to salt and they reported that the salt-tolerant cultivars seem to have better control over their stomata and maintained lower transpiration rates resultant higher photosynthetic rate. Similarly, other researchers have also reported a decrease in photosynthetic attributes (P_N, *gs, Ci, E*) in response to salt stress under salinity. Tuna et al. (2010) in mustard, Yamane et al. (2012) in rice, Ma et al. (2018) in beans, Elhakem (2020) in wheat, and Alkhatib et al. (2021) in eggplant reported that decrease in the net photosynthesis as a result of the salt stress was due to the damage to chloroplast structures.

In salt-sensitive 'Jaya,' the observed increase in the Fo and decrease in Fm in comparison to 'Korgut' indicate an organizational change in LHC, such as orientation and space between the pigment molecules and accumulation of inactive PSII reaction centers, respectively. The greater decrease in quantum efficiency of photosynthesis (Fv/Fm) ratio and photochemical quenching (qP) in 'Jaya' over 'Korgut' (Fig. 3.5.2 c,d) also suggests damage to electron transport from LHC to the reaction center and from PS II to PS I respectively. Changes in Fo and Fm value could also be due to structural damage of the chloroplast, which is indicated by the effect on the ultrastructure of cells and chloroplasts in the sensitive 'Jaya' variety (Fig. 3.3.2A; d-f), which may also affect pigment-protein complex in antenna and integrity of PS II reaction center. Our results with SDS-PAGE suggest an effect on both Chloroplast Proteins (CP) as well as oxygen evolution complex in the 'Jaya' variety (Fig. 3.10.1 a,b). However, the tolerant rice variety, which has stable Fv/Fm and Fm values, indicated a better-maintained thylakoid membrane structure (Fig. 3.3.2B; d-f) due to better ion homeostasis (Table 3.4.1). In this study, the decrease of qP was much higher in 'Jaya' (Fig. 3.5.2 d) may be due to the reduced photochemical utilization of absorbed light energy, which is also associated with the decrease in the Fv/Fm ratio. Non-photochemical quenching (qNP) increased in the 'Jaya' variety but only up to 80 mmol/l of NaCl stress and no further, but for 'Korgut,' the same was increased up to 160 mmol/l NaCl (Fig. 3.5.2 e), indicating that the activity of the xanthophyll cycle related to excess energy dissipation was enhanced more efficiently at an even higher salt concentration in 'Kogut' than in 'Jaya' again leading to better dissipation of excess energy thereby protecting the plants against ROS (Fig. 3.6.1 and 3.6.2).

Studies of salt stress on the light reaction of photosynthesis in plants have also been reported by others. Salim et al. (2021) showed that salt-sensitive genotype 'B-14011' of

barley grown at 200 mM NaCl for ten days showed a higher Fo value while exhibiting very low Fm and Fv/Fm as compared to salt-tolerant cultivar 'B-10008', suggesting limited transfer of absorbed energy from the light-harvesting complex to the reaction center and the probability of electron transport from donor end of PSII to acceptor side of PSII. In addition, the increase in Fo value could result in the separation of LHCII and PSII. Likewise, Tsai et al. (2019) noted that salt-sensitive rice variety '8777' had increased Fo and decreased Fm as compared to salt-tolerant rice variety 'IR66946', suggested changes of Fo and Fm due to the damages of chloroplasts. Liang et al. (2003) in poplar (*Populus* spp.) showed a notable decline in Fv/Fm and the Fm value, while the increase in Fo under salinity stress, indicating that PSII RCs were damaged or photo-chemically inactive and attributed to reduced capacity of PSII to transport electrons. Similarly, Ahmed et al. (2015) compared the response of salinity in Tibetan wild barley (XZ5) and cultivated barley (CM72) and observed a more significant decrease in Fv/Fm in the cultivated variety and suggested that a higher protective capacity for PSII in wild variety may be an essential tolerance mechanism for barley genotypes. A suggestion of increased qNP related to a protective mechanism to avoid ROS generation in salt-stressed plants is also reported. Lee et al. (2013), working with salt-sensitive (IR-29) and salt-tolerant (Pokkali) rice varieties, suggested that an increase in qNP in 'Pokkali' is expected to minimize photoinhibition damage to the reaction centers.

Chlorophylls and carotenoids are important photosynthetic pigments in harvesting light to chemical energy ATP and $NADPH^{+}$. We observed a higher reduction in the leaf pigment contents (chlorophyll 'a', 'b,' and carotenoids) in 'Jaya' than in 'Korgut' under salinity stress (Table 3.5.3). The observed decrease in leaf pigment contents (chlorophyll 'a', 'b,' and carotenoids) in the 'Jaya' as compared to the 'Korgut' under salinity stress can be linked to free radical-induced oxidation of chlorophyll pigment (Kato & Shimizu, 1985), interference of salt ions with pigment-protein complexes (Munns et al., 2011), disruption of chloroplast membrane (Sayyad-Amin et al., 2016) and increased activity of chlorophyll degrading enzymes, such as chlorophyllase (Parida et al., 2004) and decreased activity of Chl synthesis enzymes (Ali & Ashraf, 2011; Rasool et al., 2013). Impairment in pigment-protein complexes (Levitt, 1980), deficiency in the supply of other essential ions such as Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} that are necessary for Chl synthesis (Van Assche & Clijsters, 1990) due to salt stress may also influence pigments concentration.

A similar observation of the decline in photosynthetic pigments in plants by salinity was also reported by others. Hakim et al. (2014) reported that salt-sensitive rice varieties 'IR20' and 'BRRI dhan29' had decreased chlorophyll 'a', chlorophyll 'b,' and total chlorophyll contents than tolerant rice varieties 'MR211' and 'MR232' due to inhibitory effect of the accumulated ions on the biosynthesis of the chlorophyll fractions. Wang et al. (2013) also noted that the salt-sensitive rice variety 'Suijing NO.6' had a higher reduction in total chlorophyll content than the salt-tolerant rice cultivar 'Suijing NO.5' due to the destruction of the thylakoid membrane structure for reducing the affinity between chlorophylls and proteins of the chloroplast leading to lowered activity of chlorophyll synthesis enzymes. Ahmad et al. (2013) observed that the salt-sensitive wheat variety '11388' had a reduction in chlorophyll content (Chl 'a', Chl 'b,' and carotenoids) as compared to salt-tolerant variety '11454', accompanied by a sharp decrease in net photosynthesis *(Pn),* stomatal conductance *(gs),* and transpiration rate *(E)* in sensitive variety and suggested that photosynthetic inhibition was caused by stomatal factors and by inhibition of chlorophyll synthesis. Other researchers have also reported a decline in chlorophyll content in leaves under salt stress conditions in various plant species. Mostafa (2012) observed that the salt-susceptible genotype, *Ociumum minimum* L., of basil grown at 6 ds/m NaCl for twenty days showed reduced chlorophyll content compared to salt-tolerant *Ociumum basilicm* L. due to ROS formation. Chakraborty et al. (2016) reported that salt-sensitive *Brassica juncea* grown at 150 mM for ten days showed chlorophyll degradation than salt-tolerant *Brassica napus,* suggesting higher degradation in chlorophyll due to excessive production of reactive oxygen species in the salt-sensitive variety. Parida et al. (2004) in barley, Zeeshan et al. (2020) in canola, and Betzen et al. (2019) in wheat reported that a decrease in chlorophyll pigments is to be a result of slow synthesis or fast breakdown of the pigments in cells due to the salt stress.

4.6 Oxidative damage

We observed greater production of reactive oxygen species such as H_2O_2 and OH• in 'Jaya' than 'Korgut' on treatment with NaCl (Fig. 3.6.1 and 3.6.2), suggesting greater oxidative damage to biomolecules which was also observed in the form of peroxidation of membrane lipids, MDA formation (Fig. 3.7.1) and oxidation of protein, carbonyl formation (Fig. 3.7.3). Our findings revealed that both varieties had varying abilities to deal with oxidative stress due to their differential ability to generate ROS and metabolize it. An increase in H_2O_2 and OH• levels indicates an imbalance of various reactive species,

which may alter the membrane structure, thus affecting physiological processes, mainly photosynthesis, respiration, and ion exchange. Our data confirmed that high levels of H_2O_2 in the salt-sensitive 'Jaya' variety could accelerate processes like Haber–Weiss reaction, resulting in the formation of more hydroxyl radicals OH• than in 'Korgut,' which can cause more oxidation of proteins, lipid peroxidation, disruption of cellular membranes and disturbs the redox system of cellular and influence the main metabolic pathways by direct changes in enzyme activity and membrane properties.

The generation of ROS, in addition, to directly measuring H_2O_2 and OH•, was also indirectly established by studying the oxidative damage to the cell membrane and protein in the form of lipid peroxidation (Fig. 3.7.1) and protein oxidation (Fig. 3.7.3). The data suggest that sensitivity and resistance to salt stress in the 'Jaya' and 'Korgut' were directly related to ROS generation and oxidative effect. The salt-sensitive 'Jaya' showed a greater generation of ROS (H_2O_2 and OH•) and thereby more oxidative damage to both lipids and protein than seen for salt-tolerant 'Korgut'. The extent of lipid peroxidation is regarded as an indicator of a decrease in membrane stability. Our results also showed low carbonyl contents in the salt-tolerant variety, which may indicate the activity of a well-functioning antioxidant system (Fig. 3.8.1, 3.8.2, and 3.8.3). In contrast, increased carbonyl content was observed in the salt-sensitive variety after applying salt stress. Protein carbonyl groups are prevalent in organelles subjected to increased ROS generation and are the most commonly determined marker of oxidative stress. Increased lipid peroxidation in the salt-sensitive 'Jaya' variety in this study appears to be a key factor for increased membrane damage (electron leakage, Fig. 3.7.2); this is unlike in the salt-tolerant 'Korgut' variety, where less oxidative stress was seen, and less level of MDA was noted, and consequently, very little increase in membrane damage was observed.

ROS generation by NaCl in plants has also been reported by others. Chawla et al. (2013) showed that salt-sensitive 'MI-48' of rice grown at 100 mM NaCl for 25 days showed higher superoxide radical and H_2O_2 content in leaves as compared to salt-tolerant 'CSR-1', suggesting sensitive varieties have more oxidative damage of cell membranes, as was reflected in elevated EL and MDA levels. Esfandiari and Gohari (2017) noted that the salt-sensitive cultivar of wheat 'Darab2' showed a higher production of OH• and H_2O_2 than the tolerant cultivar Arta.' These results indicate that in 'Darab2', salinity induced oxidative damage and membrane leakage by oxidizing membrane lipids. Similarly, Yassin et al. (2019) noted that another wheat-sensitive variety 'Misr 2' grown at 150 mM NaCl also had significantly higher superoxide and hydrogen peroxide in leaves as

compared to the tolerant variety 'Sakha 95', suggesting that salt-tolerant cultivars exhibit less lipid peroxidation and ROS production (H_2O_2) compared to sensitive cultivars, which was attributed to efficient scavenging capacity of the tolerant cultivar. Oukarroum et al. (2015) stated that *Lemna gibba* grown at 400 mM NaCl had higher production of ROS after 24 h treatments compared to the control and exhibited a strong correlation between enhanced ROS and inhibition in the activities of PSI, PSII, and the photosynthetic electron transport chain. Similar to these results, increased levels of H_2O_2 and $O_2^{\cdot -}$ under salinity stress have been reported by Jiang and Zhang (2001) in corn, Kukreja et al. (2006) in peas, Nxele et al. (2017) in sorghum, and Abogadallah et al. (2010) in barnyard grass reported that salt-induced osmotic stress resulting in a shortage of available water and often caused oxidative stress to a cell that led to the generation of free oxygen radicals for channelizing excess reducing power produced because of decline in photosynthetic dark reaction (Mehler, 1951).

As a consequence of ROS generation, the subsequent oxidative stress in the form of lipid peroxidation and protein oxidation by NaCl in plants has also been reported by others. Mishra et al. (2013) provided evidence that the seedlings of a salt-sensitive 'Malviya-36' grown at 14 ds/m for 20 days had a higher lipid peroxidation and protein oxidation as compared to salt-tolerant 'CSR-27' variety and indicated as a potential biomarker of salt-induced oxidative damage to membranes, causing electrolyte leakage, loss of membrane permeability, and malfunctioning of membrane proteins and ion channels in salt-sensitive variety. Sarker and Oba (2020) revealed that salt-sensitive 'VA3' of the *Amaranthus tricolor* variety showed significantly higher production of H_2O_2, EL, and lipid peroxidation in comparison to the tolerant 'VA14' variety, observing the production of a large quantity of ROS caused the toxic effects of salt-induced oxidative stress in salt-sensitive variety. Nedjimi (2014) study with saltbush species and Turan and Tripathy (2013) with rice reported that salt stress treatment resulted in a sharp accumulation of OH• and H_2O_2 with a concomitant increase in MDA content, indicating an evident oxidative burst in the leaf tissues. Similar to our results, low MDA content is essential in terms of salt tolerance, as represented in different studies by Senadheera et al. (2012) in rice, Liang et al. (2003) in barley, and Ashraf & Ali (2008) in canola indicating that lower levels of MDA are an essential indicator of lower oxidative damage and higher growth.

These findings were further corroborated by the activity (Fig. 3.8.1, 3.8.2, and 3.8.3) and expression (Fig. 3.12.1) of antioxidant enzymes such as SOD, APX, and CAT. Our

data suggested a much higher threshold level of enzymatic and non-enzymatic antioxidant activity in the salt-tolerant 'Korgut' than in the salt-sensitive 'Jaya' (Fig. 3.8.4). This suggests a higher level of capacity for scavenging oxygen radicals and maintenance of cellular membranes under salt treatment in the tolerant variety. A decline in SOD, APX, and CAT activity in salt-stressed seedlings of the sensitive 'Jaya' variety indicated that in this cultivar, the H_2O_2 and OH• scavenging mechanism by these enzymes are less effective under salt stress. In addition to the higher threshold level of these antioxidant enzymes in 'Korgut' variety, the salt stress treatment resulted in only a slight further increase in the antioxidant enzymes in comparison to the salt-sensitive variety, suggesting inherent higher activity of these antioxidant enzymes even at low salt stress played a significant role in protection against oxidative damage. The data on the activity of antioxidant enzymes was also corroborated by the expression studies of these antioxidant enzymes in these two varieties under the salt stress condition. In parallel with enzymatic antioxidants, the observed much higher content of AsA in 'Korgut' than in the salt-sensitive 'Jaya' (Fig. 3.8.4) also suggests protection against oxidative damage, probably in the initial phase of stress before expressing antioxidant enzymes. The increment in leaf AsA and antioxidant enzyme activity in 'Korgut' showed a positive relationship with the reduced ROS and MDA contents.

Our data on antioxidants is in accordance with work carried out by other groups on salt stress. Chawala et al. (2013) observed that salt-tolerant rice varieties (Pokkali and CSR-1) grown at different salts such as NaCl, $MgCl_2$, $MgSO_4$, and $CaCl_2$ for 30 days showed enhanced activity of SOD, CAT, and APX in the leaves of as compared to salt-sensitive (MI-48 and IR-28) varieties, indicated that higher antioxidant activity which may allow salt-tolerant cultivars to survive under oxidative stress. Likewise, Borzouei et al. (2012) noted that the salt-tolerant wheat variety 'Bam' showed a remarkable increase in SOD and APX activities in contrast to the salt-sensitive 'Tajan' variety, implicating ability of 'Bam' cultivars in better coping with ROS. Kumar et al. (2021) also showed that salt-tolerant *Oenanthe javanica* variety 'V11E0022' showed higher antioxidant enzyme activities of SOD, POD, CAT, and APX, as compared to sensitive variety 'V11E0135', suggesting that higher activities of antioxidant enzymes in salt-tolerant variety reduced the H_2O_2 and lipid peroxidation levels in roots and attributed to better biomass and growth in tolerant variety.

Similarly, Zhang et al. (2014) observed that the salt-tolerant *Gossypium hirsutum* cultivar (CCRI-79) had significantly increased SOD activity in both the leaves and roots

than in the salt-sensitive cultivar (Simian 3), due to which salt-tolerant genotype had a more efficient $O_2^{\bullet-}$ radical-scavenging ability. Lin et al. (2010) also observed that the salt-tolerant tomato cultivar 'HS18' showed higher APX activity than the salt-sensitive cultivar TN66' suggesting higher APX activity reduced the accumulation of ROS and alleviated the damage to membranes and homeostasis in plant cells. Several authors have also reported the higher activities of APX and CAT in response to salinity stress. Li, (2009) in *Solanum lycopersicum*, and Sarker and Oba (2020) in *Amaranthus* reported that increased activity of APX may be associated with scavenging ROS at lower concentrations of H_2O_2, and CAT quenches a much greater concentration of H_2O_2.

4.7 Compatible solute accumulation (Proline)

The results of our investigation on proline content indicated a consistently linear accumulation with the increase in salt stress in the leaves of the 'Jaya' variety. On the other hand, the salt-tolerant 'Korgut' variety accumulated significantly less proline, up to 120 mmol/l of NaCl treatment but increased considerably at higher concentration (200 mmol/l of NaCl) compared to 'Jaya,' suggesting that the salt-tolerant 'Korgut' variety exhibited tolerance to salt up to 120 mmol/l NaCl by developing less salt-induced osmotic stress indicating a positively correlated with better plant water status up to 120 mmol/l NaCl. (Fig. 3.8.5). Our result with a high proline accumulation in the salt-sensitive 'Jaya' variety may provide protection against salt stress, but the metabolic cost seems to affect the growth (Lee et al., 2015). High proline levels are a characteristic of salt-hypersensitive plants. High accumulation of proline in leaves may indicate salt injury as opposed to salt resistance in rice variety under salinity.

Our data also corroborate that in the salt-sensitive variety, the accumulation of proline is maximum at low salt concentrations, indicating more salt-induced osmotic stress at lower concentrations which leads to a substantial lowering of RWC even at a low salt concentration at a higher metabolic energy cost, while salt-tolerant 'Korgut' showed a slow increase in the proline content at a lower level of salt stress indicating better plant water management, probably through the salt exclusion and compartmentalization (SOS) or reducing intake through non-selective cation channels (NSCC), and transporters involved in the Na^+ and K^+ from root to shoot through high-affinity K^+ transporters (HKTs), thus not affecting the salt concentration and not causing the physiological drought in leaves as seen with our data on RWC. This observation is further corroborated by our study on the expression of ion channels OsHKT1;1 and OsHKT2;4 (Fig. 3.12.3

a,b). OsHKT1;1 is a sodium importer, and its expression was much higher in 'Jaya' than in 'Korgut,' while the OsHKT2;4 is a co-importer of Na^+/K^+ which had higher expression in 'Korgut' than 'Jaya'. K^+ is an ion facilitating water uptake and probably a reason for better RWC in 'Korgut' as a result of the salt treatment, thus better growth compared to 'Jaya'. Our results suggest that the 'Korgut' variety showed rapid alterations in its osmotic adjustment to adapt to osmotic stress and maintain sufficient cellular water content up to 120 mmol/l NaCl treatment, while the salt-sensitive variety 'Jaya' showed a much lesser response, leading to a greater reduction in RWC (Fig. 3.2) and growth (Table 3.1). Proline biosynthesis is not always rapid in salt-stressed plants; the maximum proline accumulation might occur when plants are exposed to excessive salinity.

Similar observations regarding the accumulation of proline have also been reported by others. Nguyen et al. (2021) noted that wild rice 'JC 2304' grown at 150 mM NaCl had rapidly accumulated a high amount of free proline, while salt-tolerant 'Pokkali' showed a less free proline accumulation, suggesting proline is not directly correlated with salt tolerance but may help in developing the solute potential to maintain plant water status at a higher cost of energy. Similarly, Chen et al. (2007) and Widodo et al. (2009) have also observed that salt-sensitive barley cultivars 'Gairdner' grown at 320 mM NaCl had shown higher proline accumulation as compared to salt-tolerant 'Numa'; these results indicate that hyperaccumulation of known major compatible solutes in barley does not appear to play an important role in salt-tolerance, but rather may be a symptom of salt-susceptibility. Similarly, Sarker and Oba (2020) noted that the salt-sensitive *Amaranthus tricolor* 'VA3' variety of demonstrated a higher increase in proline content grown at 100 mM NaCl than salt-tolerant 'VA14', indicating that VA3 generated more ROS that was detoxified by the overproduction of proline.

On the contrary, Hasanuzzaman et al. (2013) demonstrated that the salt-tolerant rice variety (BRRI) grown at 150 mM NaCl showed comparatively higher proline content than the salt-sensitive variety (dhan49), suggested that increased proline content in the tolerant variety due to its adaptive features of higher tolerance. Other researchers have also reported that the accumulation of proline under salinity stress is to confer salinity tolerance and maintains RWC in many plants such as green gram (Misra & Gupta, 2005), canola (Xue et al., 2009), and sunflower (Huang et al., 2013), however, they did not study the comparative status of proline in sensitive and tolerant varieties.

4.8 Fatty acid and membrane stability

Fatty acids are one of the necessary components of membrane lipids and are considered essential for stress adaptation, including salt stress. Our results showed an increased level of saturated fatty acid in the 'Jaya' while the salt-tolerant 'Korgut' showed more unsaturated fatty acid (Table 3.9.1 and 3.9.2). Results showed a higher level of unsaturated fatty acids, oleic and linolenic acid, in the salt-tolerant 'Korgut' variety than in the salt-sensitive variety, suggesting a possible role for the unsaturation of fatty acids adaptation against salt stress tolerance. The same is also established by determining the membrane stability index (MSI) values for oleic and linolenic acid, which were found to be positively correlated with r^2 = 0.81 and 0.59, respectively. While, the salt-sensitive 'Jaya' variety showed a negative correlation of these unsaturated fatty acids with MSI values of r^2 = - 0.657 and -0.529 (Table 3.9.3). The 'Korgut' variety appeared to experience less oxidative damage as compared to 'Jaya,' which perhaps may be due to its superior capacity to reduce the toxic effect of ROS due to the positive correlation in ROS and 18:1ω9 and 18:3ω3 in 'Korgut' leaves, indicating their key importance in the protection against the oxidative damage. Moreover, salt-sensitive 'Jaya' seedlings showed decreased levels of 18:1ω9 when the degree of salt stress increased, whereas 18:3ω3 was present only in control ones, which suggests that alteration in both these fatty acids reflect the damage caused by salt stress in sensitive variety and also altered the nutrition value (Table 3.9.1).

A few reports have studied the comparing account of fatty acid in the salt-sensitive and tolerant variety under salinity stress. Zaman et al. (2019) observed that salt-tolerant purslane variety 'CPL' grown at 200 mM of NaCl showed a double bond index (DBI) had significantly increased as compared to the salt-sensitive 'PL,' indicating that increased unsaturation of fatty acid in tolerant variety is imperative to sustain the membrane fluidity and positively related to the plant's salt tolerance. Hajlaoui et al. (2009) noted that salt-tolerant *Zea mays* cultivar 'Arper' grown at 102 mM NaCl for 20 days showed a higher proportion of polyunsaturated fatty acids as compared to salt-sensitive 'Aristo,' indicating that linolenic and eicosapentaenoic acids were considered to be the characteristic of the relatively salt tolerance 'Arper' variety. Sui et al. (2018) noted that peanuts grown at 150 mM NaCl have a higher level of unsaturated fatty acid and suggested that unsaturated fatty acids play a crucial role in protecting cell membranes by maintaining the function of membrane proteins.

4.9 Thylakoid membrane proteins

Our results with the SDS-PAGE profile of thylakoid proteins (Fig. 3.10.1 and 3.10.2) suggested a significant loss of thylakoid proteins in the salt-sensitive 'Jaya' in contrast to the salt-tolerant 'Korgut' variety, which may lead to a decreased photosynthetic rate and eventually lower biomass. Degradation of photosystem II (D1 proteins) in 'Jaya' (Fig. 3.10.1) indicates a decrease in Fm (Fig. 3.5.2 b) and represents a disruption in energy transfer within the reaction center affecting Fv/Fm ratio. The observation also suggests a decrease in ΦPSII and that NaCl may have affected the number of functional reaction centers, which can be associated with the observed reduction in the Fm due to open reaction centers. The observed decrease in abundance of LHC (25-28 kDa) protein in 'Jaya' (Fig. 3.10.1b) may suggest the effect of NaCl on decreasing the efficiency of light harvesting and efficient transfer to the PSII RC. The structural disorganization of the LHC is also suggested by the changes in Fo (Fig. 3.5.2 a). The coupling factor (63-65 kDa) showed a slight increase in the 'Korgut' variety as a result of the NaCl treatment, suggesting that excess NaCl may not have affected ATP generation in salt-tolerant 'Korgut' variety in comparison to the salt-sensitive 'Jaya,' which showed a decreased level of coupling factor protein.

There are few reports of studies on thylakoid proteins exposed to NaCl. Zhou et al. (2015) reported that *Salicornia bigelovii*, a halophyte grown at 400 mM NaCl, led to an enhanced amount of PsaA/B, CP47, CP43, and Lhcb1 with a concurrent increase in antennae size, suggesting an increased energy transfer from the light-harvesting antennae to the photosystems and these photosystem changes may be responsible for the adaptation of *S. bigelovii* to saline conditions. Hameed et al. (2021), and Nishiyama and Murata (2014) reviewed that salinity stress inhibited protein synthesis and the degradation of the chloroplasts thylakoids (CP47) and D1 protein, which caused photodamaged PSII and decreased the Electron Transport Rate (ETR). Huseynova et al. (2016) in drought-sensitive wheat 'Garagylchyg 2' showed a reduction in 12kDa, CPI, CP47 CP43, and 32kDa protein as compared to drought-tolerant 'Barakatli 95', suggesting that there protein in drought tolerance variety has provided stability of thylakoid membranes and their electron transport chain against damaging action of free radicals. Farhat et al. (2019) showed that *Cakile maritime* (halophyte) grown at 200 mM NaCl for two months did not decrease the amount and composition in the Light-Harvesting Complex of PSII (LHCII3), the Chl 'a' protein complex containing P680 and its associated core antennae (CP43), and the light-harvesting protein complex of PSII (LHCII) which may constitute the unique

regulatory process for halophytic behavior of the species and adaptation to the saline environment.

4.10 Protein profiling (Swath Analysis)

Data observed using the proteomics approach identified the key salt-tolerance proteins by comparing the contrasting genotypes under salinity stress. The salt-tolerant 'Korgut' variety showed a 2-fold increase in the Differentially Abundant Proteins (DAPs) than those seen in salt-sensitive 'Jaya' (Fig. 3.11.2), indicating better biological, cellular, and molecular functions related to salt-tolerant in 'Korgut' and this parallelly, support physiological data observed in this study. The proteomics data suggested that the primary underlying mechanism of salt tolerance in 'Korgut' involved photosynthesis, carbohydrate, energy metabolism, antioxidant system, ion homeostasis, and plant water status.

The observed upregulation of five thylakoid membrane proteins: cytochrome b6-f complex, chlorophyll a/b binding protein, chloroplast Oxygen-Evolving Enhancer protein 1 (OEE1), ferredoxin-NADPH oxidoreductase and chloroplast 23kDa polypeptide of photosystem II, and three carbon-fixing enzymes such as Malate Dehydrogenase (MDH), and Phosphoglycerate Kinase (PGK) in salt-tolerant 'Korgut' compared to salt-sensitive 'Jaya' variety on treatment with NaCl (Table 3.11.1), mainly associated with light and dark reactions of photosynthesis, suggested that these proteins are responsible for higher photosynthesis resulting in greater productivity to 'Korgut'. The observed 3-fold upregulation of proteins associated with energy metabolism, such as Ribulose-1,5-Biphosphate Carboxylase (RuBisCO), cytosolic malate dehydrogenase, fructokinase-2, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Calvin cycle Protein (CP12), and ATP synthase in salt-tolerant 'Korgut' compared to the 'Jaya' variety (Table 3.11.1) suggest an increased accumulation of soluble sugars to provide efficient energy for plants to maintain plant growth under stress condition.

Others have reported that NaCl reduced the photosynthesis protein and affected TCA and the electron transport process in salt-sensitive plants. Jha et al. (2022) noted that salt-tolerant pearl millet 'IC 325825' grown at 150 mM NaCl showed more abundance of OEE, RuBisCO, and ATP β-synthase as compared to salt-sensitive 'IC 325825', suggesting that more abundance of these proteins in tolerant variety are required for the activity of photosystem II (PSII), cyclic electron transport, and protection of oxidoreductase enzyme from proteolytic degradation which allowing its survival and

better adaptability under salt stress. Lakra et al. (2018) observed that the salt-tolerant rice variety 'Pokkali' showed a higher abundance of PsbP/Chloroplast, 23kDa polypeptide of photosystem II, Cyt b6/f, RuBisCO, GAPDH, and GLY1 than in salt-sensitive 'IR64', suggesting that salt-tolerant by better photosynthetic machinery leading to the better growth rate. Similarly, Ghaffari et al. (2014) observed that salt-tolerant mutant rice (167-1-3) showed higher RuBisCO protein than salt-sensitive (S-730-1) and suggested that it maintained RuBisCO in an active state even at low concentrations of stromal CO_2 in tolerant variety while decreased RuBisCO expression may be one of the major factors conferring susceptibility to salinity. Likewise, Sarhadi et al. (2012) noted that the salt-tolerant rice variety 'Cheriviruppu' grown at 100 mM NaCl showed up-regulation of three isoforms of fructokinase-2 which were downregulating in salt-sensitive 'IR64'. Upregulation of fructokinase-2 was suggested to increase starch content in pollen, which supported pollen growth and development under salt stress. Hu et al. (2019) noted that *Cannabis sativa* grown under salt stress showed up-regulation of protein and higher expression of genes of CP12 and ATPase and suggested that upregulation of CP12 genes induced by salt stress might be aimed at meeting metabolic demands. Cheng et al. (2016) also observed the upregulation of CP12 in salt-tolerant hemp halophytes (BM), which was downregulated in the salt-sensitive variety (YM), indicating that CP12 protein was closely related to salt-stress tolerance.

Proteomics data also indicate more than 3-fold upregulation of antioxidant proteins such as ascorbate peroxidases, peroxiredoxin, superoxide dismutase, and glutathione reductase in salt tolerant 'Korgut' than in salt-sensitive 'Jaya' grown at the highest concentration of NaCl in this study. It was also observed that Glyoxalase I (GLY I) was only expressed in tolerant 'Korgut' but not in the sensitive 'Jaya' variety when grown at 200 mmol/l NaCl (Table 3.11.1). Our proteomic data are in consent with the higher level of gene expression of ascorbate peroxidases and superoxide dismutase in salt-tolerant 'Korgut,' thereby affirming a low level of oxidative damage and less production of ROS as compared to 'Jaya'. This could also be a reason for gaining higher productivity in 'Korgut' even at a high level of salt. In addition, the presence of GLY I in 'Korgut' indicated that the glycolytic pathway is associated with tolerance to salt stresses. The glyoxalase system has been proposed to be involved in protection against α-oxoaldehyde cytotoxicity, regulation of cell division, and proliferation. The glyoxalase system plays a major role in detoxifying Methylglyoxal (MG), a cytotoxic byproduct of the glycolytic pathway (Urscher et al., 2011). The observed increase in the protein level of glutathione

reductase in our study may also be due to its role in the glycolytic pathway to convert 2-oxoaldehydes into 2-hydroxy acids (Saadat, 1999). Ghosh et al. (2022) noted that transgenic tomatoes (salt-tolerance) had increased Glyoxalase activity and expression that resisted the accumulation of excess MG and ROS under salt stress. Gupta et al. (2018) also observed that transgenic rice plants grown at 200 mM NaCl showed overexpression of Glyoxalase I, suggesting that glyoxalase-overexpression involved improved MG detoxification and reduced oxidative damage under stress conditions.

The observed increase in Glutamine synthetase (GS) in the salt-tolerant 'Korgut' variety than in salt-sensitive 'Jaya', (Table 3.11.1), which catalyzes the ATP-dependent condensation of ammonium with glutamate to yield glutamine and provides nitrogen for the biosynthesis of all nitrogenous compounds in the plant (Skopelitis et al., 2006) and its increase indicate a unique salt-tolerant characteristic that allows plants to adapt to changing environments.

Sahu et al. (2001) observed that salt-tolerant rice varieties (Getu and CSR-3) grown at 200 mM NaCl for 21 days showed higher protein expression of GS as compared to salt-sensitive varieties (Ratna and CR 44-11), suggesting a biochemical adaptation for salt tolerance in rice. Yan et al. (2005) also noted a down-regulation of GS protein in rice (Nipponbare) grown at 150 mM, which was linked to reduced GS activity and proline production, and suggested that it might be part of the reason for its salt sensitivity.

Our data showed 14 ribosomal proteins expressed only in tolerant 'Korgut' but not in the sensitive 'Jaya' when grown under salt stress (Table 3.11.1). In addition, the eukaryotic translation initiation factor (eIF) was upregulated in tolerant 'Korgut' compared to sensitive 'Jaya' (Table 3.11.1). The presence of ribosomal and eIF proteins in 'Korgut,' indicating essential ribonucleoprotein complexes engaged in translation and thus play a crucial role in metabolism and growth. Eukaryotic translation initiation factor (eIF) proteins help in the regulation of translation, which facilitates the selective synthesis of required proteins and is one of the versatile strategies plants have evolved to cope with environmental stresses (Hossain et al., 2016).

Few studies have clarified the role of eIF in abiotic stress. Yang et al. (2017) observed that *Tamarix hispida* eIF1A (TheIF1A) grown at different abiotic stresses, such as NaCl and polyethylene glycol (PEG), showed that eIF1A protein expression is much higher in salt and drought stress in comparison to their control. They also showed the expression of eIF1A protein was more in salt-stressed than in drought-stressed plants and indicated a stress response regulator to improve plant salt and osmotic stress tolerance via

regulation of associated enzymes and ROS scavenging, thus reducing cell damage under stress conditions.

Our data also showed upregulation of Plasma membrane intrinsic proteins (PIPs) PIP1;2, PIP2;2, and PIP2;1 in 'Korgut' than in 'Jaya' when grown at 200 mmol/l NaCl (Table 3.11.1) which may help improve water uptake by roots and leaf cell hydration, which stabilizes plant water status and WUE at the cellular levels.

Similar to our results, Li et al. (2018) observed upregulation of TsPIP1;1 in mutant rice variety (*Thellungiella salsuginea, TsPIP1;1*) grown at 100 mM NaCl and suggests that overexpression of the TsPIP1;1 is involved in the regulation of water transport, the accumulation of Na^+/K^+ ions, and the translocation of photosynthetic metabolites, thus conferring enhanced salt tolerance to rice. Lian et al. (2006) also observed that rice plants (MT401), when exposed to water deficit conditions, showed the overexpression of OsPIP2 and provided better WUE, resulting in better plant growth.

Lipid transfer proteins (LTPs) were also expressed only in tolerant 'Korgut' but not in the sensitive 'Jaya' when grown at 200 mmol/l NaCl (Table 3.11.1). Lipid transfer proteins (LTPs) are small proteins that synthesize lipid barrier polymers, such as cuticular waxes, suberin, and sporopollenin, and are considered key proteins for the plant's survival under abiotic stresses (Salminen et al., 2016). Our data with LTPs suggest that the presence of LTPs in 'Korgut' may have promoted an increase in cuticle wax and the number of trichomes under salinity stress involving trapping water. There are limited reports on the role of NaCl in the upregulation of LTPs protein. Tapia et al. (2013) noted that the expression of different LTPs genes in leaf epidermis showed a positive correlation between cuticular wax deposition and their ability to bind cutin monomers (i.e., hydroxylated fatty acids). Several LTPs genes are up or down-regulated upon different abiotic stresses, including low temperature, drought, salinity, and wounding (Maghuly et al., 2009; Fleury et al., 2019).

A 3-fold upregulation of actin-binding proteins (ABPs) in salt-tolerant 'Korgut' compared to the 'Jaya' variety (Table 3.11.1) in our study in indicated that ABPs involvement in cell division and osmoregulation under osmotic stress and associated with tolerance to salt stresses. The ABPs have been proposed to play a major role in plant development by regulating several fundamental cellular processes, together with cell division, cell expansion, organelle motility, and vesicle trafficking to adjust cellular behavior to substantial quantities of salt and minimize its toxicity in plant cells growth (Paez-Garcia et al., 2018).

There are limited reports on the role of actin protein under NaCl. Askari et al. (2006) noted that *Suaeda aegyptiaca* (halophyte) grown at 600 mM NaCl for ten days showed the upregulation of actin protein, resulting in a profound reorganization of plasma membrane integrity and ion channels and may also be associated with cell expansion. Xiong et al. (2017) reviewed that enhanced ABPs levels during salt stress may result in the depolymerization of actin filaments and enhanced K^+ influx through the inward rectification of potassium channels, which may restore ion homeostasis

4.11 Transcriptome studies: In the present work, we have studied the expression of the following genes OsP5CS, Cu/Zn-SOD, APX, OsHKT1;1, and OsHKT2;4 as they are functionally involved in salt-regulated cell processes.

Proline synthesis gene (OsP5CS)

P5CS is one of the biosynthetic genes involved in the proline pathway as it produces $NADP^+$ and stimulates the pentose phosphate pathway to control redox potential in the cell. While ProDH (proline dehydrogenase) is involved in proline degradation. Our result showed a higher accumulation of proline at 200 mmol/l NaCl in 'Korgut' than in 'Jaya,' where the proline content was seen to increase throughout the stress regime (Fig. 3.8.5). The higher content of proline at the highest salt stress in 'Korgut' seems to be due to its lower degradation than seen in 'Jaya,' rather than higher synthesis of proline (Fig. 3.12.2). The main role of P5CS is argued to be more than a basic proline metabolism and is likely to help with plant water status (RWC) and membrane stability (MSI). An increase in the expression for OsP5CS in the 'Korgut' variety while less proline content explains the fact that transcriptional level is not always directly related to the enzymatic activity, and maybe this gene is differentially regulated by salinity stress. The expression patterns indicate that OsP5CS might have different functions in both rice varieties.

Expression studies of P5CS in response to NaCl have reported differential isozyme responses depending on the plant species. Hmida-Sayari et al. (2005) observed that proline overexpression is accompanied by an improved salt tolerance in the transgenic potato (proline synthesis gene) lines compared to wild-type, suggesting it is regulated by salt stress and the production of proline is energy consuming; hence, plants have developed a control mechanism to prevent its accumulation under standard conditions. Guan et al. (2020) showed that transgenic *Panicum virgatum* (*A. thaliana* P5CS) grown at 350 mM NaCl concentration showed overexpressing PvP5CS gene and exhibited

higher relative water content than control plants under salt stress, resulting in lesser membrane damage, lipid peroxidation, and higher proline levels than control plants. Kant et al. (2006) noted that *Thellungiella halophila* halophytic model species showed increased proline synthesis expression under salt stress. A Pearson correlation analysis of data showed a significant correlation between its expression and RWC, EL, and MDA under salinity stress. Verslues & Sharma (2010) in Arabidopsis grown under salt stress increased proline accumulation due to P5CS, P5CR upregulation, and PDH down-regulation. Xue et al. (2009) in *Brassica napus* showed the increased expression of isozymes BnP5CS1 (pyrroline-5-carboxylate synthetase1) and BnP5CS2 (pyrroline-5-carboxylate synthetase1), while the expression of BnPDH (proline dehydrogenase) was inhibited under salt stress, indicate that stress-induced proline accumulation in B. napus results from the reciprocal action of activated biosynthesis and inhibited proline degradation. They have also observed during development that proline content was high in reproductive organs and was accompanied by markedly high expression of BnP5CS and BnPDH, suggesting possible roles of proline during flower development in salt-grown plants. Several studies have also observed overexpression of the P5CS gene increases proline content and salt tolerance. Kaikavoosi et al. (2015) in *Oryza sativa*, Pavei et al. (2016) in *Triticum aestivum* L., Surekha et al. (2014) in *Cajanus cajan* L., and Guan et al. (2019) in *Cuccinelli chinampoensis*, suggesting that overexpressing of P5CS genes, improves salt tolerance by reducing the electrolyte leakage and ROS levels.

Antioxidant gene expression (Cu/Zn-SOD and APX)

Antioxidants are vital for detoxification and protection against oxidative damage caused by ROS accumulation under salinity stress. The higher expression of Cu/Zn-SOD and APX in 'Korgut' (Fig. 3.12.1) resulted in increased SOD activity leading to lowered ROS production and oxidative damage to the cell membrane in comparison to 'Jaya'. Overall, increased expression concurred with the increased activity of antioxidant enzymes in the 'Korgut' variety, improving its ability to limit oxidative damage corresponds to a high photosynthesis rate.

Our results of the higher expression level of antioxidant enzymes are supported by Mishra et al. (2013), who observed that salt-tolerant (CSR-27) rice grown at 140 mM NaCl for 10 days showed a higher Cu/Zn-SOD expression than salt-sensitive (Malviya) variety, and antioxidant activity. Lee et al. (2018) in seaweed, in response to UV and salt stress, observed overexpression and activity of SOD and APX, suggesting that these

genes are essential components that contribute to cell homeostasis and effectively reduce intracellular ROS levels.

Expression of OsHKT transporter genes

HKT1 transporters (subfamily I) belong to Na^+, while those in HKT2 (subfamily II) are selective for co-importer of Na^+/K^+ that is implicated in the maintenance of membrane potential and ion homeostasis under salinity (Gao & Song, 2021). Observed higher expression of OsHKT1;1 (Fig. 3.12.3 a) in salt-sensitive 'Jaya' than in salt-tolerant 'Korgut,' and OsHKT2;4 (Fig. 3.12.3 b) in 'Korgut' than 'Jaya,' suggested to result in greater accumulation of Na^+ salt in salt-sensitive variety, while facilitating K^+ retention over Na^+ in 'Korgut' thus maintaining the better regulation of K^+/Na^+ homeostasis in 'Korgut' (Table 3.4.1) which facilitated effective water uptake and probably a reason for better RWC in 'Korgut' under salt stress (Fig. 3.2).

There are only a few studies on the effect of salinity on the expression of OsHKT transporter genes. Kader et al. (2006) noted in salt-tolerant rice 'Pokkali' grown at 150 mM NaCl showed a down-regulation of OsHKT1 while upregulation of OsHKT2 as compared to salt-sensitive 'Dhan29' suggested the salt tolerance in 'Pokkali' by maintaining a low cytosolic Na^+ level and controlling a ratio of cytosolic Na^+/K^+. Similarly, Miyamoto et al. (2015) observed overexpression of OsHKT2;1 in rice plants resulting in accumulated Na^+ in roots but not in shoots, suggesting that another Na^+ transporter may regulate Na^+ transfer from root to shoot. Han et al. (2018) observed increased expression of HvHKT2;1 in the plasma membrane of the epidermis and steles in the root of barley grown under salts tress and concluded that HvHKT2;1 participated in the lateral transport of Na^+ and maintained the balance of K^+ and Ca^{2+} in the roots. Hauser & Horie (2010), Mishra et al. (2016), and Zhang et al. (2017) have also reported that OsHKT2;4 expressions contribute to a lower cytosolic Na^+/K^+ ratio and thereby reduced Na^+ toxicity.

4.12 Conclusion

Salinity is one of the significant abiotic stresses and constraints for agriculture worldwide since most crop plants are sensitive to salt stress. Thus, it is imperative to understand the different components of the salt-tolerant network in plants to produce salt-tolerant cultivars. The current study analyzed salt (NaCl) stress on the growth, physiological, biochemical, and molecular characteristics of salt-sensitive rice 'Jaya' and

salt-tolerant 'Korgut' varieties. Our work concludes that the 'Korgut' showed higher tolerance to salt stress than 'Jaya' owing to better plant water status, morphological changes such as an increased number and size of trichomes, and a decreased stomatal size. Our data confirmed the presence of Lipid Transfer Proteins (LTPs) in 'Korgut,' which promoted an increase in cuticle wax and the number of trichomes under salinity stress and prevented water loss to increase the RWC and plant water status. Upregulation of Plasma membrane Intrinsic Proteins (PIPs) may also account for better RWC in 'Korgut' to tolerate salt stress. 'Korgut,' in our study, also showed better ion homeostasis than 'Jaya' by higher uptake of K^+ in the root, which may indicate a barrier mechanism between root and shoot. A Higher K^+/Na^+ ratio and less accumulation of Na^+ ions in 'Korgut' may play a critical role in salinity tolerance through the exclusion of Na^+ and Cl^- ions from sensitive leaf tissue and maintained K^+/Na^+ for better osmoticum. The same was further confirmed with a gene expression study showing up-regulation of OsHKT2;4 (K^+ and Na^+ symporter) and down-regulation of OsHKT1;1 (Na^+ transporter) in 'Korgut' over 'Jaya'. Our proteomic data showing abundant Actin Binding Proteins (ABPs) in 'Korgut' further confirm the role of ion homeostasis by enhanced K^+ influx. Oxidative stress as a consequence of salt stress is demonstrated by the production of ROS, which leads to oxidative damage to membrane lipids and protein, more so in 'Jaya' than in 'Korgut'. The observation suggested better membrane integrity seen as a low level of lipid (MDA) and protein (carbonyl content) oxidation and electrolyte leakage in 'Korgut,' which is further confirmed by a fatty acid composition that showed biosynthesis of unsaturated fatty acid such as α- linolenic acid (18:3ω3) and oleic acid (18:1ω9), which contribute to the better maintenance of membrane integrity in the salt-tolerant variety. 'Korgut' also showed better chlorophyll a/b and carotenoids to chlorophyll ratio and better RWC, resulting in higher CO_2 assimilation and productivity. Our results with chlorophyll fluorescence also suggest a role of salinity in pigment content, composition, and orientation influencing photosynthesis. Our studies concluded a higher threshold and greater stimulation of an antioxidant enzyme which was further supported by higher gene expression of SOD, CAT, APX, and upregulation of proteins such as GLY1, GPX, and GR in the salt-tolerant 'Korgut' variety, resulting in limiting ROS-mediated oxidative damage. We conclude that a salt-induced increase in antioxidative metabolism is one of the important protective processes against ROS-mediated oxidative damage in deciding the salt tolerance level of 'Korgut'. Proteomics data also validate our physiological and

biochemical observation by indicating that proteins associated with functions suggested playing a role in salt tolerance SOD, CAT, and APX.

Based on our data, we conclude that salt tolerance to the rice variety ‘Korgut’ is a multipronged strategy at different levels like morphological adaptation leading to better plant water status, membrane stability, and ion homeostasis in order to prevent ion toxicity and maintain membrane integrity and physiological and biochemical processes leading to ROS mediated oxidative damage. Our data provide morphological, physiological, and molecular markers that can be used for developing salt tolerance variety through genetic engineering.

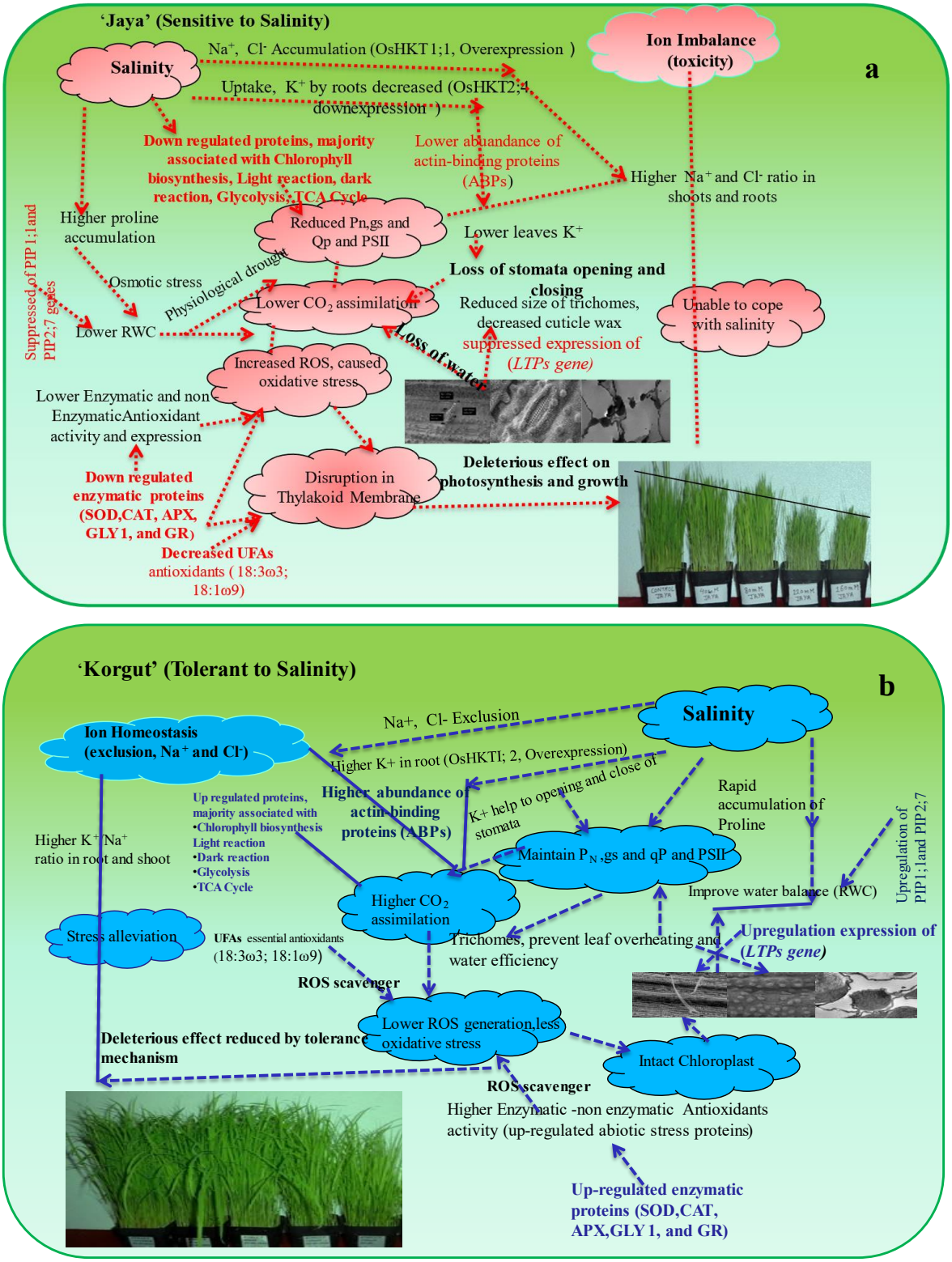


Fig. 4.12.1: A model summarizing the response of salinity in the salt-sensitive 'Jaya' (a) and salt-tolerant 'Korgut' (b) variety. The red line (a) indicates an inadequate response to salt resulting in sensitive, while the blue line (b) indicates an adequate response to salt stress resulting in salt tolerance.

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“Scientific innovations continually provide us with new means of analyzing the finds.”

Richard Leakey

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Srivastava, S., & Sharma, P. K. (2021). Effect of NaCl on chlorophyll fluorescence and thylakoid membrane proteins in leaves of salt sensitive and tolerant rice (Oryza sativa L) varieties. *Journal of Stress Physiology & Biochemistry*, *17*(2), 35-44. (UGC-CARE LIST)

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Attended seminars and conferences

"Effect of salt stress on growth, physiological, and biochemical changes in salt tolerance and salt-sensitive varieties of rice (*Oryza sativa* L.)." National Conference of Plant Physiology 2016, University of Agriculture Science, Bangalore, 10 Dec 2016.

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