

**Physio-dynamism related to phytodesalination
and phytoremediation of copper in
Volkameria inermis L.**

*Thesis submitted to
the University of Calicut in partial fulfillment of
the requirements for the degree of*

DOCTOR OF PHILOSOPHY IN BOTANY

By

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**PLANT PHYSIOLOGY AND BIOCHEMISTRY DIVISION
DEPARTMENT OF BOTANY
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Certificate

This is to certify that the thesis entitled "**Physio-dynamism related to phytodesalination and phytoremediation of copper in *Volkameria inermis* L.**" submitted by **Sarath G. Nair** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Botany** of the **University of Calicut**, is a *bonafide* record of the research work undertaken by him in this department under my supervision and guidance and no part thereof has been submitted for the award of any other degree.

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Date: 26-11-2024

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Declaration

I hereby declare that the work presented in the thesis entitled "**Physio-dynamism related to phytodesalination and phytoremediation of copper in *Volkameria inermis* L.**" is based on the original work done by me under the guidance of **Dr. Jos T. Puthur, Professor, Plant Physiology and Biochemistry Division, Department of Botany, University of Calicut** and has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis are undergone plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents.

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Dedicated to
My brother with all my heart.....

Physio-dynamism related to phytodesalination and phytoremediation of copper in *Volkameria inermis* L.

Abstract

The present study was carried out to investigate the NaCl and CuSO₄-induced physiological dynamism in *Volkameria inermis* L., a mangrove-associated plant. The healthy stem cuttings of the plants were collected from the natural habitats and grown in the polyhouse. After 80d of growth, the rooted cuttings were transferred to 300 mL half-strength modified Hoagland medium. The healthy plantlets of *V. inermis* were then subjected to varying concentrations of NaCl (0, 100, 200, 300, 400, and 500 mM) and CuSO₄ (0, 100, 150, 200 and 250 µM) for 20d under hydroponic culture conditions. From the results of the preliminary phase of the study, it was clear that *V. inermis* could effectively tolerate up to 400 mM of NaCl and 200 µM CuSO₄, and deleterious stress effects were shown in plantlets grown in concentration above it. Further detailed analysis was carried out to investigate the tolerance potential of the *V. inermis* plantlets subjected to various concentrations of NaCl and CuSO₄ in the tolerance limit. The plant growth under NaCl and CuSO₄ was assessed using various plant growth parameters such as fresh and dry weight, leaf area, tissue water content, and tolerance index. The supra optimal concentration of NaCl and CuSO₄ affects plant growth negatively and at the same time, the lower modest concentration of NaCl (100 mM) and CuSO₄ (100 µM) exhibits growth promotion as compared to control plantlets. The stress imparted by different concentrations of NaCl and CuSO₄ was evaluated by assessing the stress intensity factors such as accumulation of hydrogen peroxide (H₂O₂), superoxide (O₂^{•-}), membrane stability index (MSI), electrolyte leakage, and extent of lipid peroxidation. The exposure of *V. inermis* to higher NaCl and CuSO₄ treatments causes oxidative stress by the upregulation of the reactive oxygen species such as H₂O₂ and O₂^{•-}, which causes damages to the components of photosynthesis and affects the plant growth negatively. Hence, the photosynthetic efficacy was modulated under different concentrations of NaCl and CuSO₄. The total chlorophyll content in the leaves of the plantlets decreased with increasing concentrations of NaCl and CuSO₄ and lower concentrations of NaCl and CuSO₄ enhanced the total chlorophyll content over control. The osmotic stress induced by NaCl and CuSO₄ was countered by the upregulation of osmolytes in *V. inermis*. The

enhanced accumulation of osmoprotectants safeguards the organelles, bio-membrane, and cytosolic enzymes. The augmented antioxidative machinery including non-enzymatic antioxidants (total phenolics, ascorbate, glutathione, and flavonoids) and enzymatic antioxidants (superoxide dismutase, ascorbate peroxidase and guaiacol peroxidase) in *V. inermis* actively scavenge reactive oxygen species (ROS) and maintains the cellular homeostasis under stress. The higher bioaccumulation of sodium and copper in the root tissues and lower translocation of the same to the shoot regions proved its phytostabilization ability. The subcellular distribution studies in the different fractions of plantlets exhibit higher sequestration of Na in the soluble fraction and Cu in the cell wall fraction. The Cu ions were found to be complexed with the polysaccharides of cell walls and thereby limiting the cellular toxicity to a greater extent. The sequestration of toxic ions in the cell wall and wall debris fraction of roots reduce the metal toxicity reaching the leaves and thus protects the photosynthetic machinery. Higher level of cell wall lignification helps to sequester toxic metal in the cell wall with the support of carboxyl and hydroxyl groups of the lignin which tightly binds the Cu ions. Moreover, the higher lignin content specifically provides rigidity and hydrophobicity, which supports in encountering the metal toxicity. Higher lignified cells in the root, especially in the xylem walls, validate the Cu phytostabilization potential of *V. inermis*. The modulations in the secondary metabolites were analysed by HR-LCMS and a higher number of compounds were detected in treated plantlets, which are known to play roles in metal chelation and stress tolerance. The increased gene expression of phenylalanine ammonia-lyase and phytochelatins synthase genes shows the activation of the phenylpropanoid pathway for producing different phenolic secondary metabolites, having a role in metal chelation in *V. inermis*. This study clearly shows that *V. inermis* is a good candidate for phytodesalination and copper phytoremediation in NaCl and copper-affected areas since it is equipped with strong tolerance strategies.

Keywords: Mangrove-associate, Phytodesalination, Phytoremediation, Phytostabilization, *Volkameria inermis*

സംഗ്രഹം

'വോൾക്കമേറിയ ഇനേർമിസ്' എന്ന ശാസ്ത്രനാമത്തിലറിയപ്പെടുന്ന കണ്ടൽ സഹവർത്തിയായ പുഴമുല്ലയിൽ ഉപ്പിന്റെയും (രാസനാമം സോഡിയം ക്ലോറൈഡ്) തുരിശിന്റെയും (രാസനാമം കോപ്പർ സൾഫേറ്റ്) പ്രേരകമായി ഉണ്ടാകുന്ന ജീവൽപ്രവർത്തനങ്ങളെക്കുറിച്ചുള്ള സമഗ്രഅന്വേഷണമാണ് ഈ പഠനം. പ്രസ്തുത പഠനത്തിന് ആവശ്യമായ ചെടിയുടെ കാണഡം അവയുടെ സ്വാഭാവിക ആവാസമേഖലയിൽ നിന്നു നേരിട്ട് ശേഖരിച്ച് അവയെ കാലിക്കറ്റ് യൂണിവേഴ്സിറ്റി ക്യാമ്പസിലെ ഗ്രീൻഹൗസിൽ നിയന്ത്രിത സാഹചര്യങ്ങളിൽ വളർത്തി തുടർ പഠനങ്ങൾക്ക് വിധേയമാക്കി. പഠനത്തിന്റെ ഭാഗമായി, ഈ ചെടികളെ വ്യത്യസ്ത ഗാഢതയിൽ ഉള്ള ഉപ്പിന്റെയും (0, 100, 200, 300, 400 & 500 mM) തുരിശിന്റെയും (0, 100, 150, 200 & 250 μ M) ലായനിയിൽ വളർത്തുകയും ചെടിയുടെ ഉയർന്ന പ്രതിരോധനില (സോഡിയം ക്ലോറൈഡ് - 400 mM; കോപ്പർ സൾഫേറ്റ്- 200 μ M) നിർണ്ണയിക്കുകയും ചെയ്തു. ചെടിയുടെ വളർച്ചാപരമായ മാറ്റങ്ങളെ അവയുടെ ഭാരം, ഇലയുടെ വിസ്തീർണ്ണം, ടിഷ്യൂ ജലത്തിന്റെ അളവ്, സഹിഷ്ണുത സൂചിക, എന്നിവയുടെ അടിസ്ഥാനത്തിൽ നിർണ്ണയിച്ചു. ഉപ്പിന്റെയും തുരിശിന്റെയും ഉയർന്ന ഗാഢത ചെടിയുടെ വളർച്ചയെ പ്രതികൂലമായി ബാധിക്കുകയും അതേസമയം കുറഞ്ഞ ഗാഢത സസ്യവളർച്ചയെ ഉത്തേജിപ്പിക്കുന്നതായും കാണപ്പെട്ടു. ഉപ്പിന്റെയും തുരിശിന്റെയും വ്യത്യസ്ത ഗാഢത മൂലമുണ്ടാകുന്ന ചെടിയുടെ സമ്മർദ്ദത്തിന്റെ തീവ്രത പഠിക്കുന്നതിനായി ഹൈഡ്രേജൻ പെറോക്സൈഡ്, സൂപ്പർ ഓക്സൈഡ്, ലിപിഡ് പെറോക്സൈഡേഷൻ, ഇലക്ട്രോലൈറ്റ് ലീക്കേജ് എന്നീ പഠനങ്ങൾ നടത്തി. ഉയർന്ന തോതിലുള്ള ഉപ്പിന്റെയും തുരിശിന്റെയും സമ്മർദ്ദം ചെടിയുടെ പ്രകാശസംശ്ലേഷണത്തെ കാര്യമായി ബാധിക്കുകയും തത്ഫലമായി ചെടിയുടെ വളർച്ച മുരടിക്കുന്നതായും പഠനത്തിൽ കാണപ്പെട്ടു. സമ്മർദ്ദ ഫലമായി ഉണ്ടായ ആന്റി ഓക്സിഡന്റുകൾ ചെടിയിലെ റിയാക്ടീവ് ഓക്സിജൻ സ്പീഷീസുകളെ ഒരു പരിധി വരെ കുറയ്ക്കുന്നതിന് സഹായകമാണ്. ചെടികളുടെ മറ്റുഭാഗങ്ങളെ അപേക്ഷിച്ച് കോപ്പറിന്റെയും സോഡിയത്തിന്റെയും ഉയർന്ന ജൈവശേഖരണം വേരുകളിൽ കാണപ്പെട്ടു. വേരുകളിലെ ഉയർന്ന ശേഖരണവും കുറഞ്ഞ സ്ഥാനചലനവും

വോൾക്കമേറിയ ഇനേർമിസിന്റെ ഫൈറ്റോ സ്റ്റബിലൈസേഷൻ കഴിവിനെ തെളിയിക്കുന്നു. ഉയർന്ന ലിഗ്നിന്റെ അളവ്, സൈലം കോശങ്ങളുടെ കനം എന്നിവ വിഷലോഹങ്ങളെ ശേഖരിച്ചു വെക്കുന്നതിൽ പ്രധാന പങ്കുവഹിക്കുന്നു. എച്ച്.ആർ.ൽ.സി.എം.എസ് എന്ന സാങ്കേതികവിദ്യ ഉപയോഗിച്ച് വിവിധ ഇനം രാസപദാർത്ഥങ്ങളെ തിരിച്ചറിയുകയും അവയുടെ പട്ടിക തയ്യാറാക്കുകയും ചെയ്തു. ചെടിയുടെ ജൈവപ്രവർത്തങ്ങളിൽ ഈ രാസ പദാർത്ഥങ്ങൾ കാര്യമായ പങ്കു വഹിക്കുകയും ഇവ ഉയർന്ന സമ്മർദ്ദത്തെ കുറയ്ക്കുന്നതിനായി പ്രവർത്തിക്കുന്നുണ്ടെന്നും കണ്ടെത്തി. മോളികുലാർ ജീൻ എക്സ്പ്രഷൻ പഠനത്തിലൂടെ ഫീനൈൽ അലാനിൻ അമോണിയ ലയാസ്, ഫൈറ്റോ കീലറ്റിൻ സിന്തെസ് എന്നീ ജീനുകളുടെ പ്രവർത്തന ക്ഷമത അളക്കുകയും അവയുടെ ഉയർന്ന പ്രവർത്തന ക്ഷമത വോൾക്കമേറിയ ഇനേർമിസ് എന്ന സസ്യത്തിന്റെ സമ്മർദ്ദത്തെ കുറയ്ക്കുന്നതായും പ്രസ്തുത പഠനം തെളിയിച്ചു. മേൽ നടത്തിയ പഠനങ്ങളുടെ അടിസ്ഥാനത്തിൽ വോൾക്കമേറിയ ഇനേർമിസ്, ഉയർന്ന അളവിലുള്ള ഉപ്പിനെയും തുരിശിനെയും ചെറുക്കുന്നതിന് മികവുറ്റ ഒരു ചെടിയാണെന്ന് തെളിയിക്കപ്പെട്ടു.

സൂചകപദങ്ങൾ: കണ്ടൽ സഹവർത്തി, ഫൈറ്റോഡെസലൈസേഷൻ, ഫൈറ്റോറെ മീഡിയേഷൻ, വോൾക്കമേറിയ ഇനേർമിസ്, ജൈവശേഖരണം.

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ABBREVIATIONS

ABA	-	Absciscic acid
ABS	-	Absorption flux
APX	-	Ascorbate peroxidase
AsA	-	Ascorbate
CAT	-	Catalase
Chl	-	Chlorophyll
CK	-	Cytokinin
CSI	-	Chlorophyll stability index
C _{Sm}	-	Cross section related to F _m
Cu	-	Copper
CuSO ₄		Copper sulphate
DCMU	-	3(3,4dichlorophenyl)-1,1-dimethyl urea
DCPIP	-	2,6-dichlorophenolindophenol
DHA	-	Dehydroascorbate (oxidized ascorbate)
DHAR	-	Dehydroascorbate reductase
D _{Io}	-	Dissipated energy flux
DTNB	-	5-dithio-bis-2-nitrobenzoic acid
DW	-	Dry weight
EL %	-	Electrolyte leakage %
E _{To}	-	Electron transport flux
E _{TR}	-	Electron transport rate
F _m	-	Maximum chl <i>a</i> fluorescence
F _o	-	Initial chl <i>a</i> fluorescence
FTIR	-	Fourier Transform Infrared
F _v	-	Variable chl <i>a</i> fluorescence
FW	-	Fresh weight
GCMS	-	Gas chromatography and mass spectrometry
GSH	-	Glutathione
H ₂ O ₂	-	Hydrogen peroxide
HEPES	-	N-(2-Hydroxyethyl)piperazine-N-(2-Ethanesulphonic acid)

HR LC-MS	-	High Resolution Liquid Chromatography-Mass Spectrometry
IBA		Indole butyric acid
KBr	-	Potassium bromide
LHC	-	Light harvesting complex
MDA	-	Malondialdehyde
MDHA	-	Monodehydroascorbate
MDHAR	-	Monodehydroascorbate reductase
MSI	-	Membrane stability index
MT	-	Metallothionein
MV	-	Methyl viologen
Na	-	Sodium
NaCl	-	Sodium chloride
NaN ₃	-	Sodium azide
NBT	-	Nitroblue tetrazolium
O ₂ ^{•-}	-	Superoxide
pBQ	-	para-Benzoquinone
PCs	-	Phytochelatins
PCS	-	phytochelatin synthase
PI	-	Performance index
POD	-	Guaiacol peroxidase
PSI	-	Photosystem I
PSII	-	Photosystem II
R	-	Pearson's correlation coefficient
RC	-	Reaction center
ROS	-	Reactive oxygen species
RWC	-	Relative water content
SEM	-	Scanning Electron Microscope
SOD	-	Superoxide dismutase
TBA	-	Thiobarbituric acid
TCA	-	Trichloroacetic acid
TF	-	Translocation factor
TRo	-	Trapping energy flux

1. INTRODUCTION

In nature, plants are constantly confronted with varying stress factors, which may include both biotic and abiotic ones. Primary biotic agents of stress include disease-causing pathogens and attacks by herbivores. In contrast, abiotic factors include salinity, toxic metal ions, drought and flood, radiations, thermal fluctuations, radionuclides, etc. (Chang et al., 2020, Praveen et al., 2023). According to recent records, more than 60% of crop yield worldwide is impeded by various abiotic stressors (Kopecká et al., 2023). This may seriously affect the nutritional demand of the ever-increasing population, which may turn to 10.4 billion people by 2100, as per the recent statistical analysis (United Nations, 2023).

Plants are the most fruitful candidates in the ecosystem, having the inherent ability to counteract abiotic stresses by developing various adaptive strategies. Breeding abiotic stress-tolerant plants is a milestone for sustainable agricultural practices. A meticulous understanding of the defense strategies, including elucidating the stress-responsive signaling pathways, is mandatory (Napier et al., 2023). Recent developments in this field include characterizing the abiotic stress-tolerant phenotypic traits and improving tolerance with tools like omics, CRISPR-Cas9, etc. On the other hand, abiotic stress factors may also considerably deteriorate the soil's quality and structure to a greater extent and make the soil unfit for the growth of plants.

1.1 Land degradation

Efficient management of soil health is one of the prime focuses of researchers and must be addressed to ensure productivity and profitability. The quality of arable land is declining at an alarming rate, and the primary

reason for the rapid decrease is the presence of various abiotic agents like excess salt ions, toxic metal ions, radionuclides, poor oxygen levels, etc. (Oleńska et al., 2020).

1.1.1 Salinity induced land degradation

Soil salinization poses a severe threat to most plants and is induced by the accumulation/precipitation of soluble salts in the soil (Seleiman et al., 2022). Recently, Praveen et al. (2023) have reported that about 831 Mha of land have turned out to be unproductive due to increased salinity across the world, and this may result in massive loss of agricultural space, which in turn leads to crop loss, amounting to US\$ 27 billion/year. The increased salt load in the soil is mediated by the unavailability of fresh water, sea level rise, backwater intrusion, irrigation of crop plants with salt/wastewater, changing rain patterns, drought conditions, unscientific drainage systems, etc. (Alkharabsheh et al., 2021; Al-Suhaibani et al., 2021; Badawy et al., 2021; Kamra, 2021). Increased alkalinity/sodicity in soil may hamper the quality of soil mainly because of the presence of abundant electrolytes, uneven distribution of Na^+ and Ca^{2+} in the soil solution, the declining organic content of the soil, rhythmic transformation of the hydrological cycle, poor vegetation and less biological activities, etc. (Basak et al., 2022).

1.1.2 Heavy metal-induced land degradation

The chemistry and structure of soil particles greatly influence the ecological function of soil. The presence of excess toxic metal ions in the soil resulting from industrial activities, burning of fossil fuels, uncontrolled use of fertilizers and pesticides, mining, etc., may lead to drastic changes in the soil chemistry, which turns out to be harmful to the existence of living beings (Dubey et al., 2018a; Praveen et al., 2023). Presence of excess toxic metal ions like Cu, Cd, As, Ni and Pb in soil affects the biological activity

adversely and is persistent, too, i.e., microbial/chemical activities do not degrade heavy metals. Soil distribution of metal ions may be influenced by various processes like plant uptake, ion exchange, complexation, precipitation, adsorption/desorption, immobilization, etc. (Wuana and Okieimen, 2011).

Copper is an essential microelement and is in third position in its usage as a metal worldwide. Excess Cu salts in soil may negatively affect the quality and productivity of soil as Cu is more tightly bound to the soil organic matter when compared to other metal ions. Copper generally alters soil chemistry by changing the pore size and organization, which may significantly affect other essential features of soil like filtering and water holding capacity (Paradelo et al., 2013). Copper exists in soil as divalent ions (Cu^{2+}) and is found to be associated with negatively charged groups (Mengel et al., 2001). The level of Cu in soil generally ranges between 1-40 ppm. However, the available Cu in the soil solution is comparatively less.

The texture and type of soil greatly influence the Cu concentration and accumulation capacity. For example, clay soils have a higher Cu retaining property when compared to sandy soils. Likewise, the solubility and availability of free Cu ions are also affected by the pH, i.e., an increase in pH decreases the solubility of Cu ions or increases the Cu holding capacity of soil particles, making it unavailable for plants. The ideal pH for maximum solubility of Cu is attained at pH-5.5 (Martinez and Motto, 2000).

1.2 Sodium as a nutrient and toxicant

Sodium is not an essential element and is classified as a beneficial or functional nutrient. Even though it is not necessary for all plants, it can aid in plant growth and may even be obligatory for some species. Terrestrial plants do not require Na^+ for either growth and development or

reproduction. One exception is a subset of C_4 plants where Na^+ has been demonstrated to be crucial. In C_4 plants, the conversion of pyruvate to phospho-enol pyruvate is facilitated by Na^+ ions. In C_4 plants like *Atriplex* spp., *Kochia childsii*, and millets, Na^+ is required as a trace element because it is essential for the transport of pyruvate into chloroplasts via Na^+ -pyruvate cotransporter (Furumoto et al., 2011). In other plants, this function is achieved by H^+ coupled pyruvate carrier. So, in this particular set of plants, Na^+ is considered as an essential element (Maathuis, 2009).

A positive response towards Na^+ absorption in plants is found during the K^+ deficiency. Because in hydrated conditions, Na^+ and K^+ show structural similarity. Increased Na^+ use could lessen the crop's reliance on costly potash fertilizer in soils where K^+ is sparse. Moderately saline soils may benefit from phytoremediation by absorbing Na^+ at the root-soil interface. Numerous crops, including asparagus, barley, broccoli, and beetroot, have been demonstrated to taste better when tiny quantities of Na^+ are present in the growth medium. The positive correlation between Na^+ in soil and halophytic plant growth is well studied. The upper limit of the Na^+ level varies according to halophytic plants and is always higher than that of glycophytes (Maathuis, 2014; Evelin et al., 2019; Isayenkov and Maathuis, 2019).

Even though the low-level Na^+ is beneficial, the excess quantity makes the soil infertile and can harm the plants. One of the main abiotic constraints limiting agricultural production worldwide is soil salinity. The glycophytes have a shallow threshold of Na^+ than halophytes. In both cases, above the limiting concentration, the growth is retarded. The primary morphological damages associated with saline stress are leaf and stem scorching, leaf abscission and senescence, and root and shoot development suppression. The higher concentration of Na^+ and Cl^- results in the reduction

of water potential and thereby causes osmotic stress. The higher increase of Na^+ in the cells destroys the ion homeostasis, affecting cellular biochemistry. The higher Na^+ and Cl^- content causes ionic stress, affecting the enzyme kinetics, bio-membranes, and nucleic acids. Reduction in chlorophyll pigments, stomatal conductivity, inhibition of photosystems, enhanced reactive oxygen species (ROS) production, and resulting oxidative damages were the significant consequences in plants on exposure to higher sodium chloride (NaCl) concentrations (He et al., 2009; Singh and Jha, 2016; Bhat et al., 2020).

1.3 Copper: An essential trace element

Oxygen has gradually accumulated in the environment due to increased photosynthetic species on Earth. This oxidative environment caused the development of iron oxides, which reduced the solubility of iron and it also lead to the the progressive liberation of soluble Cu (II) from insoluble Cu sulphide salts. Since then, Cu, which can serve similar activities, has gradually replaced iron in biological components. This explains why many Cu-proteins have a functional equivalent that needs Fe as a cofactor and why development on a substrate with a hazardous Cu level is typically connected to a decreased Fe content in roots and leaves (White and Broadley, 2009; Printz et al., 2016).

In the Earth's crust, copper occurs naturally in abundance at a rate of 60 mg/kg. The typical daily recommended intake of copper for adults ranges from 0.2 mg for babies to 1.3 mg for lactating women. Due to the low concentration of copper in edible plant tissues, deficiencies of Cu can arise. The plants generally growing in calcareous or alkaline soils of arid and semi-arid climates are deficient in Cu. Copper is an essential trace element in plants and has many significant roles. The required amount of Cu in plants is meager (<0.01 % of dry weight) and is also categorized as a micronutrient

(Williams and Salt, 2009). The copper-containing abundant protein is plastocyanin, a significant component of photosynthetic electron transport. The copper plays a significant role in Cu/Zn superoxide dismutase (SOD) of cytoplasm, peroxisomes, and chloroplast stroma, which participate in antioxidation mechanisms. Additionally, the role of Cu has been expanded to include cytochrome c oxidase, plastocyanin oxidase and reproductive stages development, ethylene sensing, cell wall metabolism, amino oxidase, laccase, polyphenol oxidase, iron mobilization, protein trafficking, etc. (Yruela, 2009; Adrees et al., 2015; Shabbir et al., 2020).

Excess or lack of Cu hurts vital physiological processes in plants, which can lead to abnormalities in the development and growth of plants. Copper deficiency generally appears first in younger leaves, which undergo chlorosis, distortion, or even necrosis, and reduced copper seriously affects several enzyme functions and the proper physiological status of plants. Similarly, excess Cu, exceedingly toxic, results in symptoms like chlorosis and necrosis, stunting, and suppression of root and shoot growth. The abundance of Cu in the cells cause the unavoidable binding with sulfhydryl groups of proteins and inhibits its activity, disturb ion homeostasis, hamper cell transport mechanisms, and cause oxidative damage (Marastoni et al., 2019; Rehman et al., 2019; Kumar et al., 2021a).

1.4 Toxic effects of excessive copper

Copper is a vital metal that is crucial in plants and animals. The optimal quantity of Cu is necessary for proper cellular functions. The excess/deficiency of Cu negatively impacts normal homeostasis and even affects plants' survival. Cu is a redox-active metal and can exist in two oxidation stages, Cu^{2+} and Cu^{+} . This property of Cu can be helpful in many electron transport reactions, but at the same time, the redox activity may also encourage the creation of reactive oxygen radicals. So, the content of copper

in cells needs to be strictly regulated (Shabbir et al., 2020). The excess amount of Cu in humans causes Kayser–Fleischer (KF) rings on the inner surface of the cornea, commonly found in patients with Wilson disease (a genetic disorder that prevents the body from removing extra copper). The other symptoms of this disease include wheezing, rales, lung damage, dyspnoea, erythema, etc. (Brewer, 2010).

The Cu concentration above 20-30 $\mu\text{g/g}$ in the soil causes toxicity, and at the same time, 1-5 $\mu\text{g/g}$ of Cu is essential for proper plant growth. The essential and the toxicity concentrations vary according to the plant species. In plants, the excess amount of Cu negatively affects growth, and the symptoms include reduced root growth and leaf area, wilted leaves, interveinal foliar chlorosis, low branching of roots, rupturing of root epidermis and cortex, bronzing and necrosis of leaf tips (Chen et al., 2015a). A higher amount of Cu in the soil drastically affects root architecture. The root hair formation and distortion of the entire root structure occur with a high amount of Cu in the soil. The 3.0 mM copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) treatment in lentil plants reduced the root and seedling growth and also caused a reduction in the plant biomass (Hossain et al., 2020).

The excess Cu can affect the bio-membrane by producing ROS and causing severe oxidative stress. The photosynthetic machinery is ultimately affected in response to higher levels of Cu. The chlorosis due to Cu causes a reduction of photosynthetic pigments, thereby yellowing of the leaves. The structural variation of chloroplast, inhibition of photosystem II (PSII), and the hindrance in integrating pigments with photosystems generally occur in response to the higher level of Cu. Due to the higher production of ROS, the membranes become destabilized, including thylakoid membranes. This affects the electron transport system, causing decreased photosynthesis (Küpper et al., 2009; Gonzalez-Mendoza et al., 2013). Under Cu treatment in

soils, there is a decrease in photosynthetic rates and PSII quantum yield and an increase in initial and maximum fluorescence. Along with the decline in photosynthetic pigments, inhibition of PSII reaction centers also occurs (Batool et al., 2015). The nutrient misbalancing due to excess Cu can affect the normal ionomics of plants and cause severe phytotoxic effects. The higher reductions in Fe, P, and K were prominent, and a disproportion in absorption of other nutrients was also observed.

1.5 Plant tolerance mechanisms

Higher NaCl and Cu concentrations cause abnormalities in the physiological state of plants, which in turn slows down their growth. High NaCl and Cu concentrations trigger an oxidative burst by amplifying the generation of ROS. This affects the cell membranes, DNA, RNA, proteins, and all other organic components. The oxidation of such components results in severe damage, even leading to the death of the plants (Habiba et al., 2015; Marques et al., 2018; Hussein and Alshammari, 2022). The activation and upregulation of stress-responsive genes and antioxidation mechanisms, including enzymatic and non-enzymatic antioxidants, protect plants from these stressors to a certain extent. The role of non-enzymatic antioxidants like glutathiones, ascorbate, phenolics, flavonoids, polyphenols, anthocyanins alkaloids, etc., gets enhanced in response to stressors, and it helps to effectively detoxify the ROS (Chandrasekhar and Ray, 2017; Saleem et al., 2020a; Sidhu et al., 2020).

Plants exposed to NaCl and Cu stress experience an increase in their antioxidative defense mechanism due to the formation and build-up of ROS. A short change from the optimal level activates protective genes, increasing the activity of antioxidant enzymes like catalase (CAT), SOD, ascorbate peroxidase (APX), and guaiacol peroxidase (POD), which are necessary for neutralizing free radicals and preventing the damaging effects of stress.

Antioxidant enzymes are thought to moderate oxidative damage, protecting biomolecules from stressors. Plants have inherent potential towards heavy metal detoxification (Miteva et al., 2010; Navarrete et al., 2019). They utilize different mechanisms, including blocking and trafficking of metal ion uptake, producing osmoprotectants, and augmenting metal-binding molecules like metallothioneins (MTs), phytochelatins (PCs), etc. The structural modulation in plant anatomy, including variation in stomatal and trichome index, increased circularization of the epidermis, lignification of xylem vessels, and deposition of cell wall materials, are effective adaptive strategies against NaCl and Cu stresses. In response to the NaCl and Cu, the activity of the phenylalanine ammonia-lyase (PAL) enzyme is enhanced, and it turns out to be helpful in the synthesis and accumulation of secondary metabolites in plants. PAL is critical in secondary phenylpropanoid metabolism (Sharma et al., 2016; Chaudhary et al., 2018; Wei et al., 2022; Zhang et al., 2022).

1.6 Phytoremediation and Phytodesalination

Phytoremediation, green remediation, or agro-remediation is a broad technical term for decontaminating toxicants from polluted soil or water using green plants. Plants detoxify or sequester the contaminant within the plant body, thereby remediate the contaminated site. Plants utilize different mechanisms for remediation such as degradation (rhizo-degradation, phytodegradation), accumulation (phytoextraction, rhizofiltration), dissipation (phytovolatilization), and immobilization (hydraulic control and phytostabilization). The mechanism adopted by plants varies according to the species and type of contaminant in the soil or water. Phytoextraction is the most popular method for removing pollutants from soil among these types of phytoremediation. Plants absorb, transfer, or sequester the toxicant within the plant root or shoot tissues (Saleem et al., 2020b; Kafle et

al., 2022). A good phytoremediation plant exhibits a potent tolerance towards heavy metals and produces an increased rate of biomass even under constraints; it also has other properties of being easy to grow, having good root architecture and absorption rate, and being non-attractive to herbivores. In some cases, the remediation of toxicant from the contamination site by plants may be a difficult process, mainly due to the unavailability of the toxicant in the absorbable form. So, in such cases, some other substances are used to enhance plants' phytoremediation potential. Such substances used as soil amendments are generally called chelants and are of different types. These help the plant to absorb the toxicant by increasing the bio-availability, decreasing the toxicity of contaminants, or increasing the growth of plants (Ashraf et al., 2019a; Chengatt et al., 2022).

Phytodesalination is the term specifically referring to the remediation of Na^+ from saline soil. The technique is similar to that of phytoremediation. The Na^+ is absorbed from the saline soil and then translocated into the harvestable part of plants or sequestered within the root. Generally, Na^+ -hyperaccumulating plants such as halophytes are used for this purpose because halophytes are more tolerant than glycophytes under saline conditions. The phytodesalination potential varies from species to species (Hasanuzzaman et al., 2014a; Sarath et al., 2021). Mangroves are potent halophytes with higher phytodesalination potential than mangrove associates. There are three different types of halophytes, each exhibiting a different tolerance level to high salinity. The first class of halophytes, known as euhalophytes, have succulent leaves and stems and can accumulate ions in vacuoles. However, pseduohalophytes use their innate system to prevent salts from interfering with their growth in saline soil, and they have low levels of Na^+ in their upper biomass compared to their lower biomass. Recretahalophytes use specialized structures like salt glands and bladders to protect themselves against salinity stress. By excreting the absorbed salts

outside, it helps to lower the plant component's salt level (Song and Wang, 2015; Yuan et al., 2016). The first and most crucial phase in the phytodesalination process involves identifying the candidate most suited for the salinized soil. The plants adopted for this purpose ought to exhibit the traits listed below;

- High salinity tolerance potential
- Significant biomass production
- Capacity to sequester sodium ions in the biomass
- High economic value after the desalination process and harvest (like fodder, fuel, essential oil, etc. (Sarath et al., 2021)

Traditional remediation techniques used to restore heavy metal/salinity-affected land have several drawbacks, including high costs, adverse effects on soil microbiota, complicated implementation procedures, low effectiveness, etc. As a result, it is now extremely important to develop and implement a cost-effective approach for the proper and sustainable management of salinity/heavy metal-affected soil and its recuperation by natural means. Halophytes are utilized for this purpose and are considered as a successful bio-remediating agent. Even in challenging environments, halophytes have efficient physiological and molecular pathways for quick growth (Jesus et al., 2015; Farzi et al., 2017). Green technologies such as phytoremediation and phytodesalination technology have received much attention in this twenty-first century because of eco-friendliness, simple to administer, and low cost.

1.7 *Volkameria inermis*; a boon to degraded land

Volkameria inermis L. (synonym: *Clerodendrum inerme* (L.) Gaertn). It is an evergreen straggling shrub in the family of Lamiaceae that grows

abundantly in the backwaters of low-lying areas and rocky coastal belts and is categorized as a mangrove associate and a halophyte. Mangrove associates are suitable for studying the mechanisms of adaptive evolution in plants because they represent a typical lifestyle between terrestrial plants and true mangroves. Plants that grow on the landward edge of mangrove ecosystems, often in non-mangrove habitats like salt marshes, are known as mangrove companions. Because of their ability to endure high salinity, many mangrove companions are categorized as halophytes. The potential of these plants for salinity and heavy metal tolerance needs to be well studied. The phytochemical studies revealed that it is a promising medicinal plant with a broad spectrum of pharmacological activity that could be used in various medical applications. The main pharmacological effects of *V. inermis* include anti-cancer, anti-inflammatory, antioxidant, hepatoprotective, analgesic, antibacterial activity, anti-tyrosinase, antifungal, neuroprotective, hypotensive, hypoglycemia, amyloid-aggregation, wound healing, antipyretic, and larvicidal activity (Srisook et al., 2015; Sarath et al., 2024).

Extensive study is being carried out on the plants used for the green remediation techniques and the mechanisms by which tolerance is conferred. In this scenario, halophytes like *V. inermis*, affected by soil salinity and heavy metal toxicity, would be an exciting subject for thoroughly examining the inherent tolerance mechanisms and potential to successfully in the soils affected with salinity and heavy metals toxicity. Understanding the fundamentals of NaCl/CuSO₄ tolerance in halophytes and discovering novel genes from halophytes imparting tolerance will help in breeding or genetic alteration for salinity/heavy metal tolerance. Scanty reports and studies are available on the phytoremediation and phytodesalination aspects of *V. inermis*. The evaluation of physio-dynamism related to salinity and CuSO₄ tolerance potential of *V. inermis* will help to utilize the same for phytoremediation and phytodesalination. Therefore, this study envisages a

detailed analysis of the functional and structural biology of a mangrove associate plant, *V. inermis*, in response to different concentrations of NaCl/CuSO₄.

The current study was designed with the following objectives.

1. To find out the heavy metals concentration in the natural habitat of *V. inermis*
2. To analyze the stress-imparting concentration of NaCl and CuSO₄ in *V. inermis* grown under hydroponic culture conditions.
3. To assess the modulations of photosynthetic efficiency of *V. inermis* in response to different concentrations of NaCl and CuSO₄.
4. Evaluation of Na and Cu bioaccumulation in different parts of *V. inermis*.
5. Analysis of the stress intensity factors, metabolites, and antioxidative mechanisms of *V. inermis* in response to NaCl and CuSO₄.
6. Evaluation of ionomics in response to NaCl and CuSO₄ in *V. inermis*.
7. Analysis of the anatomical modulations in response to NaCl and CuSO₄ in *V. inermis*.
8. To find out the subcellular distribution of Na and Cu in the leaves and roots of *V. inermis* in response to NaCl and CuSO₄.
9. Analysis of secondary metabolites of *V. inermis* in response to NaCl and CuSO₄.
10. Gene expression analysis of phenylalanine ammonia-lyase (PAL) and phytochelatinsynthase (PCS) in both the roots and leaves of *V. inermis* in response to higher concentrations of NaCl and CuSO₄.

2. REVIEW OF LITERATURE

2.1 Mangroves and mangrove associates

One of the most prolific ecosystems on the planet is mangrove forests. These are the dominant coastal ecosystems, producing enormous amounts of organic carbon, playing an essential role in global carbon cycling, protecting and stabilizing ecosystems, and acting as sources of commercial forest products (Kathiresan and Bingham, 2001; Zhu and Yan, 2022). The flowering trees and shrubs that make up mangroves are specially adapted to the marine and estuarine tidal conditions, making them one of the world's most important coastal ecosystems. Mangroves are widely known for their physiological and morphological adaptations to deal with salinity and routine tidal inundation, particularly their specific eco-physiological features. They can excrete salt via specialized salt glands, which allows them to endure extreme salinity. As a result of their environmental adaptations, mangroves serve as an ideal model for the study of ecological adaptation to various abiotic stresses. Because of their capacity to absorb and hold heavy metals, they can prevent the spreading of heavy metal contamination in coastal areas and can be used as a phytoremediator (Srikanth et al., 2016; Sarath et al., 2022). Mangroves have a complex root architecture that helps them anchor properly into the water-logged areas, which act as a substrate for other species to attach to them and form favorable conditions (Kon et al., 2010). They are also involved in improving the water quality, thereby alleviating water pollution and the subsequent occurrence of algal blooms (Wu et al., 2008).

Mangrove forests are disappearing alarmingly and at a faster rate across the globe. The appropriate use and maintenance of this particular plant community depends on recognizing and valuing the significance of

mangroves to the coastal ecology. The mangrove habitats are currently under copious challenges, including encroachment, habitat fragmentation, deforestation, environmental pollution, sedimentation, and more. The vast diversity of mangroves must be preserved through various conservation strategies, including stringent preservation of mangrove areas. However, recent studies have shown that due to growing government and public appreciation of the ecosystem services mangroves provide, the pace of loss of mangrove forests worldwide has decreased in the twenty-first century (Friess et al., 2020). A global awareness is necessary to denote the importance of eco-restoration of mangroves because the conservation aspects vary widely in different regions (Walker et al., 2022).

There are two categories of mangrove species: true mangroves and mangrove associates. Alterations in the ecological and physiological adaptations to live in the mangrove environment have made differences in the features of mangroves and mangrove associates. True mangroves are obligate or strict mangroves classified as 'exclusive' plants adapted to mangrove ecosystems. However, the mangrove associates are regarded as 'non-exclusive' that can live in both terrestrial or aquatic ecosystems and are adapted to mangrove ecosystems. Due to their unique characteristics, mangrove associates are also called semi mangroves or back mangroves (Parani et al., 1998; Wang et al., 2011).

2.2 Climate change and soil salinity

Climate change is a severe issue with a heavy impact on the global environment. The significant effects of the temperature increase are rising sea levels, increased flooding, salt water intrusion, drought, extreme weather patterns, etc. Changes in the climate affect the weather pattern and speed up the sea level rise. Among the different factors, temperature increase due to global warming has a severe impact on sea level increase,

and it leads to the inundation of seawater into coastal land, thereby salinizing and contaminating the soil (Dasgupta et al., 2015; Velautham et al., 2019). United Nations Intergovernmental Panel on Climate Change report states that global temperature will increase by 2.6°C by 2050, and the sea level will rise by 1 m. The increase in temperature is becoming a significant concern not only for climatologists but also for plant scientists worldwide.

Adding to the problem described above, overuse of the groundwater for irrigation purposes and the increased evaporation rate will also result in soil salinization. Bennett et al. (2009) and Okur and Örcen (2020) reported that an increased evaporation rate causes a steady increase of salt in water sources and soil of arid and semiarid regions. Moreover, the shortage of good quality water for irrigation compels the farmers to use saline water for this purpose, which also causes soil salinization (Chandio et al., 2013). The incredible shoot up of greenhouse gases in the last few decades has caused a considerable increase in global warming, altering precipitation patterns. It results in changes in soil properties such as soil temperature, composition of soil gases, biological parameters of the soil, character of the litter horizon, salt content, soil organic matter content, etc. According to Saqib et al. (2019), about 10% of the land area is degraded annually due to salinity. It may be due to several reasons, such as climate change, high surface evapotranspiration (caused by increased temperature and low precipitation), natural rock weathering, and intensive use of saline water for irrigation.

2.3 Heavy metal pollution

Presently, the combined effect of climate change and pollution due to anthropogenic activities adversely affects the flora and fauna of the biotic system and also results in the total imbalance of the biome. The over pollution load on water, air, and land leads to modifying its natural

characteristics. It thereby causes an impact on organisms, starting from microforms to higher animals (Yang et al., 2018). Depositing municipal solid waste into the land or landfill management has more pronounced effects on public health. The noxious and toxic compounds, in the form of gases or solids or high concentrations of heavy metals, persistently remain in the environment, and these potent chemicals also leach out into the ground and contaminate the water column (Han et al., 2019). The pollution due to toxic metals is hastened daily because of its higher production rate through human activities than natural ones (Damore et al., 2005). The most commonly occurring lethal metal detected in contaminant areas is lead, and the other common metals are arranged according to their lethality depending on their abundance: chromium>arsenic>zinc> cadmium>copper. Owing to the persistency of lethal metals, once they reach the soil, water, or air, they can take many decades to get detoxified. Bioaccumulation and biomagnification capacity of toxic metals can reduce agriculture production, which can badly affect the economic growth of poorly developed countries.

Heavy metals are a critical pollutant due to their persistent nature and are generally at higher levels in wetland sites. Anthropogenic activities like urban and agricultural overflow, industrial effluents, boating, mining, chemical spills, sewage treatment plants, leaching from domestic garbage dumps, and mining operations have increased the content of toxic metals in wetlands (Sruthi et al., 2017). Based on their solubility under physiological conditions, 17 heavy metals may be necessary to organisms and ecosystems. Of these, Iron (Fe), Molybdenum (Mo), and Manganese (Mg) are essential as micronutrients. Zinc (Zn), Nickel (Ni), Copper (Cu), Vanadium (V), Cobalt (Co), Tungsten (W), and Chromium (Cr) are toxic elements with high or low importance as trace elements. Arsenic (As), Mercury (Hg), Silver (Ag), Antimony (Sb), Cadmium (Cd), Lead (Pb), and Uranium (U) have no known

functions as nutrients but are toxic to plants and microbes (Nies, 1999; Keeling et al., 2003; Pinto et al., 2015).

Since ancient times, copper has been a very usable substance since it is highly conductive, malleable, robust, and adaptable. It is considered an essential plant micronutrient and plays a vital role in CO₂ assimilation and ATP synthesis (Demirevska-Kepova et al., 2004). Copper is necessary for proper development and growth of plants even in deficient concentrations, but at greater concentrations, it is poisonous and results in stunting and the inhibition of root and shoot growth, as well as chlorosis and necrosis (Yruela, 2009). As a result of human demands, the usage of items made of copper and copper alloys in construction, industrial machinery, transportation, communication, and electrical and electronic gadgets has substantially expanded. Between 2012 and 2017, the global production of copper increased from 16,692 kilotons to 20,029 kilotons (Punia, 2021). Both natural and man-made processes release Cu into the environment. Mining operations, producing metal and electrical products, residential and agricultural usage of insecticides and fungicides, leather processing, and manufacturing car brake pads are all anthropogenic sources of copper released into the environment. Volcanic eruptions, wind-borne dust, and forest fires are all examples of natural causes of copper pollution. (Wei et al., 2008; Benimeli et al., 2010; Lutts and Lefevre, 2015).

Copper is a trace metal that is needed by the majority of organisms in natural ecosystems. However, it is also a common and dangerous pollutant in high quantities and can harm humans, animals, and plants (Khademian et al., 2020). Additionally, copper can be accumulated in human beings via the food chain, which can result in several health issues, such as chronic anemia, headaches, liver and kidney damage, and neurodegenerative disease. The drinking water standards established by the World Health Organisation

(WHO) in 2011 state that the highest permissible content of copper in drinking water is 2.0 mg/L. However, copper levels in water exposed to any sources of copper are typically much higher than this. Therefore, reducing copper contamination is crucial for the long-term survival of humans (Zhang et al., 2019; Dong et al., 2020).

2.4 Green remediation techniques

2.4.1 Phytodesalination

Salinization of the fertile land is a natural process occurring for various reasons and is unavoidable due to the drastic changes in the environmental conditions (Islam et al., 2019). Phytodesalination is the plant based biological approach that aims to restore the sodic and saline soil using halophytes (Devi et al., 2015). A mechanism to reclaim these lands and convert them back into fertile land is the main objective of phytodesalination (Rabhi et al., 2015). To achieve this task, halophytes are grown in these lands, and for this, potential candidates of halophytes are selected in accordance with the salinity level of the soil. Its repeated cultivation in salinized areas will reduce the salinity in the soil and lead to the growth of glycophytic plants.

Similarly, the eco-restoration of underutilized saline coastal lands with halophytic mangrove plants also has great importance. The plants used for this purpose should be able to accumulate Na^+ ions in their plant body (Rabhi et al., 2009). They also improve leaching and reduce the soil pH, and it can also result in the increased dissolution of CaCO_3 and make more Ca^{2+} available for exchange with Na^+ ions in soil (Rasouli et al., 2013). Accumulators such as halophytes and recretohalophytes have significant potential to extract salts from the soil (Jesus et al., 2015). Therefore, this group of plants is recommended for soil desalanization (Manousaki and

Kalogerakis, 2010). The halophytic plants have undergone several alterations in physiological, biochemical, and molecular mechanisms to tolerate the effects of salinity (Zhang et al., 2016). It has a higher photosynthetic rate than glycophyte under saline conditions. The higher potential of ion compartmentation, effective osmotic adjustment, and capacity to regulate salt influx are more advanced salinity tolerance mechanisms in halophytes than in a glycophyte (Parida and Jha, 2010a).

Based on the salt demand of halophytes, they are classified into three major types: obligate, facultative, and habitat-indifferent. Obligate halophytes grow only in saline habitats and show sufficient growth under saline habitats. Chenopodiaceae members fall under obligate halophytes. Facultative halophytes can withstand saline habitats and show maximum growth in soils devoid of salts. The members of Poaceae, Cyperaceae, and Brassicaceae and plants like *Aster tripolium*, *Glaux maritima*, and *Plantago maritima* come under the category of facultative halophytes. Habitat indifferent halophytes are indifferent in their habitats, even when grown in saline and non-saline conditions. The plants such as *Chenopodium glaucum*, *Myosurus minimus*, *Potentilla anserine*, *Salsola drummondii*, *Zygophyllum qatarense*, and *Aeluropus lagopoides* come under the category of habitat indifferent halophytes (Aslamsup et al., 2011; Hasanuzzaman et al., 2014b).

Yensen and Biel (2008) classified the halophytes into three categories based on their salt remediation potential, and they include excluders, accumulators, and conductors. The excluders prevent salt entry into tissues due to the salinity tolerance mechanism, and examples include *Bruguiera gymnorhiza*, *Ceriops candolleana*, *Kandelia candel*, and *Rhizophora mucronata*. Accumulators absorb salts and deposit them in their tissues without affecting the plant's normal metabolism. Moreover, examples include

Excoecaria agallocha, *Lumnitzera racemosa*, *Pentatropis sianshoides*, *Salvadora persica*, *Sonneratia apetala*, *Sesuvium portulacastrum*, and *Suaeda nudiflora*. The conductor plants remove the salts from the plant body through salt glands. It absorbs the salt from the soil and excretes it outside the plants. Halophytes, such as *Avicennia alba*, *A. marina*, *A. officinalis*, and *Aegiceras corniculatum*, can excrete salts through their foliar glands. Ravindran et al. (2007) reported the halophytic plants with desalination potential. It includes various species such as *Suaeda maritima* Dum., *Sesuvium portulacastrum* L., *Clerodendron inerme* Gaertn., *Ipomoea pescaprae* Sweet., *Heliotropium curassavicum* L., and *Excoecaria agallocha* L. Islam et al. (2019) conducted a hydroponics experiment to desalinize the saline water by culturing *Ipomoea aquatica*, *Alternanthera philoxeroides* and *Ludwigia adscendens*. Results from the study showed that *I. aquatica* was the best among the three plants with high phytodesalination capacity (130 kg Na⁺ ha⁻¹) than *A. philoxeroides* (105 kg Na⁺ ha⁻¹) and *L. adscendens* (80 kg Na⁺ ha⁻¹). The previous report of Chaudhri et al. (1964) concluded that *Suaeda fruticosa* removes 2400 lbs (1088.6 kg) salts from one-acre salt affected land. Xu et al. (2019) conducted a greenhouse experiment and suggested the two native North American plants, *Puccinellia nuttalliana* (alkali grass) and *Typha latifolia* (cattail), for phytodesalination purposes. Both these halophytic plants have the potential to extract the Na⁺ and Cl⁻ from the soil and store it in the biomass of the plants.

Halophytes remove the excess salt through salt glands, filtration of salt at the root plasma membranes, compartmentalize the excess salts in the vacuoles, and encounter salt toxicity by accelerating the synthesis of osmolytes and also by the stimulation of enzymatic and non-enzymatic antioxidants (Parida and Jha, 2010a). The phytodesalination potential of *S. oleracea* was studied in detail by Muchate et al. (2018). The salt treated *S. oleracea* showed increased activity of antioxidants, osmolytes, Na⁺/K⁺ ratio,

and accumulation of bioactive secondary metabolite (20-Hydroxyecdysone) and enhanced sequestration of Na^+ in the vacuoles than control plants. The significant increment in these biochemical and physiological processes helps this plant to withstand the high salinity. After the phytodesalination with *S. oleracea*, the desalinized soil supports the growth of glycophyte(s), which could be a crop like rice.

Rabhi et al. (2009) conducted a field experiment at El Maleh lagoon, a salt-affected ecosystem 35 km northeast of Tunis, Africa. This experiment showed that Na^+ ions significantly accumulated in *Arthrocnemum indicum* shoot and were effective in the desalinization of saline soil. Messedi (2005) also reported the tolerance and high growth potential of *S. portulacastrum* at higher salinity. Ecological restoration of the salt affected regions with *S. portulacastrum* was an effective method for desalination of saline soil. The mangroves (*Lumnitzera* and *Excoecaria* species) tolerate high salinity by sequestering the ions in the vacuoles and by cuticular transpiration (Aziz and Khan, 2001). According to the reports of Palliyath and Puthur (2018), *B. cylindrica* is a facultative mangrove with the potential to survive both in fresh water and saline habitats, and it exhibits tolerance potential even under high concentrations of NaCl (600 mM). The accumulation of metabolites such as amino acids, total phenols, and proline and activation of antioxidant enzymes (SOD, CAT, GPX, and APX) in *B. cylindrica* help them to survive in higher salinity.

There are various examples of plants having the capacity to survive in saline soils, which can be utilized for desalinization. Septiana (2019) reported that *Vigna marina* is a salinity-tolerant legume plant widely used as a productive legume in coastal areas. *V. marina* produces more antioxidants (vitamin C, anthocyanin, and alkaloids) to withstand the effects of salinity. Hence, it is considered a promising candidate for food sources in coastal

areas. The salinity tolerance potential of the halophytic grass species *Leptochloa fusca* (Kallar grass) under different concentrations of NaCl (0–600 mM) was reported by Mohammadi et al. (2019). *L. fusca* showed tolerance towards high salinity by adjusting their physiological and biochemical features, achieved by activating specific salt tolerance genes. The plant can tolerate up to 450 mM NaCl without affecting its normal physio-chemical characters. However, higher salinity induces the augmentation of antioxidant enzymes and expression of SOS1, NHX1, and plasma membrane H⁺-ATPase to withstand the NaCl stress (Badran et al., 2015). *Puccinellia nuttalliana* and *Poa juncifolia* are the two halophytic grass species that have the potential to tolerate 300 mM of NaCl without affecting the physiological processes such as photosynthesis, transpiration rate, gas exchange and leaf water content etc. (Vaziriyeganeh et al., 2018). Panda et al. (2019) suggested the xero-halophytic plant *H. salicornicum* as a candidate for saline soil reclamation. Because it has the potential to tolerate higher salinity (400 mM) by maintaining regular physiological characters such as water status, ion homeostasis, and redox status. Proper growth, powerful antioxidant machinery, and protection of photosynthetic apparatus under salinity stress and its high tolerance index indicate the potential of a halophyte for usage in saline soil reclamation.

2.4.1.1 Biological mechanism for salinity tolerance

Plants, especially mangroves or mangrove associates, deploy strategies like avoidance, resistance, or tolerance to cope with excess salt (Nikalje et al., 2019). The salt tolerance mechanism may involve specific physiological and biochemical alterations in the plants that enable the plant to maintain the capability of the protoplasm to accumulate the salt ions inside the cells. Salt tolerance in halophytes can be accomplished either by salt exclusion or salt inclusion, and salt tolerant plants utilize the energy for

the exclusion of excess salt in order to protect themselves from the toxic effects of excess salt (Ashraf et al., 2006). Salt avoidance may involve structural and physiological adaptations to exclude salt through the root membrane or minimize the salt concentrations in the cell. To cope with hypersaline conditions, mangroves adopt various strategies, namely, salt exclusion, salt accumulation, and salt secretion.

2.4.1.2 Salt excluders

Salt excluders are plants that effectively exclude most ions into soil solution or accumulate more ions in root or root shoot junctions. Inside the root, the uptaken Na^+ and Cl^- ions are sequestered in the vacuoles of parenchyma cells or may be complexed with the xylem vessels of the stem (Munns, 2005). In addition, Na^+ and Cl^- ions may be localized in the phloem cells, casparian strip, and pericycle cells of root tissue (Munns et al., 2006). Generally, the root acts as the sink for these toxic ions, and hence, the shoot system is devoid of toxicity due to the least accumulation of salt ions (Chen et al., 2018). For example, in the common reed plant *Phragmites communis* L., most of the salt ions absorbed are retained within the root tissue rather than translocating to the shoot system (Matsushita and Matoh, 1991; Chen et al., 2018). However, about 98% of salt is excluded from the soil, and only 2% is retained in the root tissue (Husain et al., 2004). The entry of salt ions to the shoot is prevented by apoplastic barriers like casparian bands, suberin lamellae, endodermis, and exodermis (Krishnamurthy et al., 2011). Several studies have revealed the role of apoplastic barriers in limiting the entry of salt ions to the shoot, like mangroves, rice, maize, wheat, sorghum, olive, etc. (Ochiai and Matoh, 2002; Zhu, 2003).

2.4.1.3 Salt secretors

Many halophytes have specialized structures called salt glands and salt bladders for excreting the excess salt from the tissue and thereby

enhancing salt tolerance by limiting the deteriorative effects of excess salt ions due to the presence of the same within the cells (Yuan et al., 2016). Salt glands are a significant strategy for plants to cope with higher salinity by excreting salts from their plant body. Salt glands are generally found on the leaves of plants grown in saline habitats (Jiang et al., 2017). The halophytic plants with salt secreting glands on their leaves are commonly called exorecretohalophytes, and those with bladder cells are called endorecretohalophytes (Yuan et al., 2016). Deng and co-workers (2015) found that the salt glands in *Limonium bicolor* have an essential and more significant role in salt tolerance. Salt glands are of four types: salt bladders, multicellular salt glands, bicellular salt glands, and unicellular vacuolated secretory hairs (Dassanayake and Larkin, 2017). Semenova et al. (2010) reported that *Distichlis spicata* showed higher salt secretion ability, and this was achieved through a unique anatomy of leaf blades, and the salt was secreted as crystals through the leaf margins. *Aegiceras corniculatum* is a mangrove plant that secretes salt through the salt gland, an essential tolerance mechanism adopted by this plant to survive in high salinity (Parida and Jha, 2010b). Liphshitz and Waisel (1982) and Breckle (2009) reported several plants with active salt secreting glands. It falls in the genera *Acanthus*, *Avicennia*, *Aegiceras*, *Aegialitis*, *Aeluropus*, *Andropogon*, *Bruguiera*, *Brachiaria*, *Bouteloua*, *Buchloe*, *Ceriops*, *Chloris*, *Coelocaryon*, *Crypsis*, *Cenchrus*, *Chrysopogon*, *Coix*, *Distichlis*, *Dactyloctenium*, *Dinebra*, *Dichanthium*, *Digitaria*, *Echinochloa*, *Erianthus*, *Frankenia*, *Glaux*, *Hyparrhenia*, *Ipomoea*, *Laguncularia*, *Limonium*, *Rhizophora*, *Reaumaria*, *Setaria*, *Sorghum*, *Sporobolus* and *Sonneratia*.

It has been reported that the cuticle of salt glands contains ferulic acid (primary phenylpropanoid), which is involved in the process of salt secretion and can be easily detected by autofluorescence (330-380 nm) analysis (Deng et al., 2015; Yuan et al., 2016). Ferulic acid provides rigidity to the cuticle and has antioxidant potential. Even though salt glands are

particular to Na^+ and Cl^- ions, in addition to salt ions, it is observed that some selected anions and cations are also secreted through salt glands (Feng et al., 2015). However, reports are stating the active secretion of these ions into the vacuole and the energy requirement for the process is met by glandular cell respiration (Hasanuzzaman et al., 2014a).

2.4.1.4 Salt accumulators

Halophytes that store excess salt in the vacuole without affecting the cellular metabolism of plants are termed salt accumulators and categorized as euhalophytes. The functioning of cells with excess accumulation of Na^+ and Cl^- ions is not hampered as the level of K^+ and Mg^{2+} ions in the cytoplasm are regulated effectively so that the activation of various enzymes is not hindered (Flowers and Colmer, 2015). For instance, halophytes like *Kalidium foliatum* and *Suaeda salsa* are salt accumulators in which the cell turgidity is maintained by the accumulation of NaCl in the vacuole and accordingly accumulate water in the cytoplasm thereby providing succulence in the leaves and stem (Song and Wang, 2015). This succulence in response to salinity is also driven by the increased carbon resources and osmolyte accumulation like proline, glycine betaine, polyphenols, soluble sugars, and inorganic ions, which may reduce the water potential resulting in enhanced levels of water uptake and storage (Song et al., 2009).

2.4.2 Phytoremediation

Phytoremediation is an eco-friendly approach to remediate the soil from contaminants and involves much less manpower. Mangroves are a particular plant well adapted to the wetlands and flourish in this ecosystem. Heavy metal contamination of the wetland is a severe issue, and its management with these unique plants is a viable solution. Mangroves adopt various strategies to handle the heavy metals in their growing environment.

2.4.2.1 Strategies of phytoremediation

2.4.2.1.1 Phytostabilization

Phytostabilization is the process by which metal tolerant plants minimize the mobility or bioavailability of the metal in the environment, thereby reducing the migration of these toxic metals into the groundwater or the food chains (Trivedi and Ansari, 2015). It helps to sequestrate (binding and sorption) and immobilize the metals so that it is converted to unavailable forms for plants (Cheraghi et al., 2011). Basharat et al. (2018) reported that, during phytostabilization, plants change the soil chemistry, and it helps in the absorption and precipitation of heavy metals in soil. The specialized redox enzymes are excreted by the plants into the soil and converts heavy metals into less toxic form (Ali et al., 2013). Above all, the immobilization of the toxic metal within the roots (through the pectins of the cell wall) and with the negatively charged cytoplasm-membrane surface due to the high electrochemical potential is another way of detoxifying metals in plants (Rengel and Zhang, 2003). The phytostabilization potential of *A. ilicifolius* concerning the metal Cd was reported by Shackira and Puthur (2017). The absorbed Cd majority was deposited in the root cortical cells, preventing the translocation into the shoot. Their work has proved this plant as a suitable one for Phytostabilization.

2.4.2.1.2 Phytoextraction

Phytoextraction involves using plants to facilitate the removal of metal contaminants from the soil matrix. In practice, metal-accumulating plants are seeded or transplanted into metal polluted soil. The roots of established plants absorb metal elements from the soil and translocate them to the above-ground shoots, where they accumulate. After sufficient plant growth and metal accumulation, the above-ground portions are harvested

and removed, resulting in the permanent removal of metals from the site. In some cases, valuable metals can be extracted from the metal-rich ash and serve as a source of revenue, thereby offsetting the expense of remediation (Cunningham and Ow, 1996). Phytoextraction should be viewed as a long-term remediation effort, requiring many cropping cycles to reduce metal concentrations to acceptable levels.

Phytoextraction applies only to sites with low to moderate metal pollution levels because plant growth is not sustained in heavily polluted soils. As a plant-based technology, the success of phytoextraction is inherently dependent upon several plant characteristics. The two most important characters include the ability to develop high biomass and to accumulate large quantities of environmentally essential metals in the shoot (Blaylock et al., 1999). Other desirable characteristics for the plant with phytoremediation potential are tolerating difficult soil conditions (e.g., pH, salinity, soil structure, and water content), a dense root system, ease of care, and establishment.

2.4.2.1.3 Phytovolatilization

Phytovolatilization is another mechanism observed in mangroves, by which the toxic metal is absorbed by mangroves from the soil and converted into volatile forms. It involves metal absorption and volatile degradation of the heavy metal and release of this into the environment through transpiration along with water vapor (USEPA, 2000). Highly toxic metals like Hg, Se, and As are absorbed and converted into less toxic volatile forms through phytovolatilization (Cristaldi et al., 2017). The plants capable of phytovolatilization have strong sulfur metabolism capacity, which helps convert toxic metals into volatile forms (Banuelos et al., 2015; Khalid et al., 2017). The absorption of highly toxic Hg and phytovolatilization of less toxic Hg was reported in *Pteris vittata* (Sakakibara et al., 2010). Three processes

are involved in this method: first, contaminants from the soil are absorbed by plants; second, the contaminants are transformed into volatile molecules; and third, the volatile chemicals are released into the atmosphere (Jan et al., 2014).

2.5 Effects of soil salinization and heavy metals in halophytes and its tolerance mechanisms

Climate change and anthropogenic activities have resulted in rising sea levels, directly reflected in the wetland and the mangrove ecosystem (Dasgupta et al., 2015). Mangroves and halophytes have the potential to tolerate the higher salinity levels and heavy metals by modulating their defense mechanisms for a better existence in salt/heavy metal degraded soil. Both the stressors alter the activities of the enzymes responsible for nucleic acid metabolism, negatively affect protein metabolism, interrupt the hormonal balance, and decrease the mobilization of seed reserve food (Khan and Rizvi, 1994; Dantas et al., 2007; Gomes-Filho et al., 2008). Besides, these stressors interrupt the structure and functioning of enzymes, damage the organelles, affect Photosynthesis, respiration, and energy metabolism, and cause membrane damage (Ramoliya and Pandey, 2002).

Salt/heavy metal tolerance is a plant's ability to live and survive under high concentrations of soluble salt or heavy metals. It can complete its life cycle under this stress condition. It involves a complex machinery of biological processes (Nasrin et al., 2019). After perceiving the stress signal, the intra cellular signaling cascades are activated, which can trigger the expression of proteins involved in stress response and other defense mechanisms, in turn helping the plants to cope with these stress conditions (Rakhmankulova et al., 2015).

2.5.1 Seed germination under high salinity

Seed germination is the most critical stage of the plant life cycle, and it is negatively affected by salinity (Anaya et al., 2018). The salinity inhibits the germination of seeds due to the lower osmotic potential of saline water, which alters the imbibition of seeds, affecting the mobilization of reserve food materials and the proteins in the seeds. The increased salinity affects seed germination, whereas lower salinity causes dormancy in halophytes. The germination of the seeds of halophytes such as *Atriplex stocksii*, *Arthrocnemum macrostachyum*, *Haloxylon stocksii*, *Suaeda fruticosa*, *Desmostachya bipinnata*, and *Aeluropus lagopoides* was inhibited at increasing salinity. Among these, *A. stocksii* and *S. fruticosa* were highly influenced by increasing salinity, showing a decrease in the rate of germination (Khan et al., 2006). The seeds of mangrove plants can either germinate under high concentrations of salinity or, in some cases, may start their germination whenever the salinity gets reduced (after rain). After being shed in the low saline habitats, the low salinity imparts an osmo/halo priming effect on seeds that positively influences germination (Gul et al., 2013). Melendo and Giménez (2019) conducted a germination experiment with the seeds of the moderate halophytic plant *Limonium supinum* at different concentrations of NaCl (0, 100, 150, 200, 400, and 600 mM NaCl). It was concluded that *L. supinum* seeds can germinate even at 400 mM NaCl, but maximum germination was shown in 100 mM NaCl.

The halophytes use innate or enforced dormancy as an essential adaptive mechanism that prevents the seeds from germinating in high saline conditions (Ungar, 1991, 1995). Seeds of halophytic plants also showed dimorphism or polymorphism, which includes the variation in the size of seeds, seed coat pattern, weight, etc. (Mohammed and Sen, 1988). The polymorphism in seeds of *Trianthema triquetra* was well studied by

Mohammed and Sen (1988), in which the seeds collected from extreme saline areas differed in seed coat pattern, weight, size, viability, and germination behavior as compared with seeds of the plants grown in less saline areas. Similarly, the seed polymorphism in size (heavier and lighter) was also found in different halophytic plants such as *Salvadora persica*, *Jatropha curcas*, *Cyperus rotundus*, and *Salicornia europaea* in response to salinity (Ungar, 1987; Dagar et al., 2004).

The high saline nature of the soil creates seed banks that help the halophytic species to withstand hyper saline conditions by maintaining the seeds in a non-germinated state (Ungar, 1995). The term seed bank is the term used to describe the non-germinated seeds remaining in the soil for a long or short time to overcome unfavorable soil conditions (Ungar, 1995). So, the seed bank state is an avoidance mechanism of halophytes to overcome high salinity by delaying seed germination. Seed banks in the soil play an important role in determining species' spatial and temporal distribution in inland saline environments and tidal coastal salt marsh habitats (Gul et al., 2013).

Mangroves have a unique feature called vivipary, in which the seeds of the mangroves germinate before shedding. It helps these plants to tolerate hypersaline conditions (Primavera et al., 2019). After being shed into saline water, the seeds with radicles quickly establish their roots on a suitable substratum. So, this viviparity helps the mangroves to flourish easily in saline soil habitats (Kumari et al., 2020). *Aegiceras corniculatum*, *Avicennia marina*, and members of the family Rhizophoraceae showed vivipary as the mode of germination. The mangroves such as *Bruguiera cylindrica*, *B. gymnorrhiza*, *B. parviflora*, *B. sexangula*, *Rhizophora apiculata*, *R. mucronata*, and *R. stylosa* also showed viviparity as a mode of germination (Dagar, 1987; Dagar and Sharma, 1989).

2.5.2 Seed germination under heavy metals

Germination of seeds and formation of seedlings are highly sensitive to heavy metal pollution (Sanjose et al., 2022). Many authors have reported that germination and growth of seedlings are dependent on the concentration of heavy metals in their growth medium (Kranner and Colville, 2011; Asati et al., 2016). Plants exhibit various mechanisms and strategies as a defense against these metals. Even during germination, defense responses like reduced uptake of metals, chelation of heavy metals, or the initiation of antioxidant activities are exhibited (Kranner and Colville, 2011). Several halophytes show these mechanisms, making them capable of germinating and surviving under heavy metal pollution (Thomas et al., 1998; Van Oosten and Maggio, 2015; Goyal et al., 2020). A study by Márquez-García et al. (2013) reported that Cu did not exert a detrimental effect on seed germination in the halophytes *Atriplex halimus* and *Salicornia ramosissima*. The germination and seedling growth of *Spartina maritima*, a halophyte, was also unaffected by Cu (Infante Izquierdo et al., 2020).

According to the study conducted by Curado et al. (2010), *Spartina densiflora* seeds were found to germinate in acidic and heavy metal polluted sediments of the Tinto river (South West Iberian Peninsula). The sediment analysis from the site revealed that these areas are degraded with heavy metals such as Cu, Zn, Pb, Al, etc., and they are too acidic (pH 2). The heavy metal entry in to the seeds during the hydration process may be reduced by seed coats in such cases (Munzuroglu and Geckil, 2002). However, several studies have reported a decreased rate of germination under Cu stress in various plants (Singh et al., 2007; Sfaxi-Bousbih et al., 2010; Pena et al., 2011). This decrease was observed due to inhibiting the isoenzymes alpha-amylase and invertase, resulting in the restricted breakdown of starch and sucrose (reserve food resources).

Moreover, Cu stress changes the metabolism and water transport processes during the germination of seeds (Sethy and Ghosh, 2013). Cu toxicity may also lead to the abnormal growth of radicle and plumule during the germination of seeds (Nijara et al., 2019). Germination of seeds of different plants was found to be negatively affected at Cu concentrations above 80 mM, but in some plants, it was above 300 mM or even over 1000 mM (Peralta et al., 2001; Ahsana et al., 2007; Singh et al., 2007). The low germination potential at a high concentration of Cu is a method of tolerance and the concentration at which the seed germination was inhibited varied in different plants. The seeds of *Lactuca sativa* exhibited low germination in 300 μ M, *Alyssum murale* in 500 mg/kg, *Glycine max* in 300 mg/L and 1000 mg/kg (Nicholls and Mal, 2003; Brinza et al., 2018; Ganesh and Sundaramoorthy, 2018; Shams et al., 2018). The low germination rate of seeds under Cu was found in plants like *Lactuca sativa*, *Alyssum murale*, *Glycine max*, and *Lythrum salicaria*.

2.5.3 Photosynthesis under salinity

Salinity induces structural modifications on chloroplast, such as damage to thylakoid membranes and dissociation of light harvesting complex II and PSII reaction centers (Yamane et al., 2004; Yamane et al., 2008). The alterations in the photosynthetic characters were observed by analyzing chlorophyll fluorescence. The F_0 (minimal fluorescence) was increased in salt treated plants, and at the same time, F_m (maximal fluorescence) was reduced in salt treated plants (Sarkar et al., 2013). The maximum quantum efficiency of the salt affected plants' PSII (F_v/F_m) was reduced even under moderate salinity (Lee et al., 2013; Singh and Sarkar, 2014). The alterations in these parameters indicate that the salinity increase in soil negatively influences photosynthesis and, thereby, plant productivity. High salinity (300–400 mM NaCl) induced reduction of parameters such as

rate of Photosynthesis (PN), stomatal conductance (g_s), rate of transpiration (E), water use efficiency (WUE), quantum yield of PSII (Φ PSII), photochemical quenching (qP), and electron transport rate (ETR) in xero-halophyte *Haloxylon salicornicum* was reported by Panda et al. (2019). Trotta et al. (2012) reported that the C_3 halophytic plant *Arthrocnemum macrostachyum* showed reduced PSII activity and grana unstacking when it was grown without salt, and these plants exhibited increased PSII activity and improved grana formation on adding NaCl up to 1M. The reduction of pigments during salt stress is a common symptom of plants grown under high saline conditions, and the photosynthetic responses of the plants under salinity stress conditions can be correlated with the reduction in the pigments. The salinity causes the inactivation of the enzymes for chlorophyll biosynthesis and enhances the expression of chlorophyllase, thus reducing the pigments and photosynthetic efficiency (Santos, 2004). Enzymes and proteins are sensitive to high salt concentrations. Upon increased salt stress, the ions entering the cell interfere with proteins and enzymes associated with Photosynthesis, reducing their functionality (Flowers and Colmer, 2015).

Hossain et al. (2010) also reported that abiotic stress, including salinity, enhances (2-6 fold) the content of the toxic compound methylglyoxal. However, accumulating the same at lower concentrations is not toxic and can act as a signaling molecule. Methylglyoxal is a dicarbonyl derivative, a toxic compound produced mainly from triose phosphate (glycolysis) and lipid and protein metabolism. It has the potential to degrade proteins by modifying amino acids such as arginine, lysine, and cysteine, forming imidazole derivatives, deactivating antioxidant machinery and DNA damage by reacting with guanyl nucleotides to form imidazolinone adducts (Yadav et al., 2005; Rai et al., 2019).

Salinity stress negatively affects the process of Photosynthesis in plants, but halophytes and mangroves have mechanisms to cope with this increased salinity. They immediately sense the increased salinity level in the soil and modulate their physiological and biochemical activities. The salt tolerant *B. parviflora* accumulates proteins, osmolytes, and antioxidants in response to higher salinity levels (400 mM NaCl) (Parida et al., 2002). The oxidative stress caused by salinity stress leads to photosynthetic inefficiency due to thylakoid membrane damage, lipid peroxidation, activated chlorophyllase activity, etc., and plants tolerate these stress effects by activating their antioxidants (enzymatic and non-enzymatic), which scavenges the ROS (Rajaravindran and Natarajan, 2012). Activation of genes to produce antioxidants in response to salinity helps the plants tolerate high salinity and avert damage to photosynthetic machinery (Ozgur et al., 2013). Hence, in most cases, a slight reduction in photosynthetic performance might be attributed to the activation or rerouting of energy to the antioxidant defense strategies.

Furthermore, there were no negative impacts on chloroplast morphology in the halophyte *Suaeda physophora*, and the ultrastructure of chloroplast appears intact even when exposed to a higher concentration of 700 mM NaCl (Zhang et al., 2010a). The greater tolerance of halophyte chloroplasts to excess salt ions could be due to the selective exclusion of salt from the cytoplasm and subsequent storage in the vacuole, which reduces the cytoplasmic toxicity of excess salt ions (Shabala et al., 2013). A better knowledge of the chloroplast genome in halophytes could open novel ways to improve crop salinity tolerance. To date, more than 50 salt responsive genes have been characterized from the chloroplast genome of halophytes, which includes proteins involved in the organization of thylakoid membrane, PSII regulation, assimilation of CO₂, photorespiration, ABA

metabolism and signaling, ion homeostasis and scavenging of ROS developed under excess salt ions (Rabhi et al., 2012).

Under high salt concentrations inside the mesophyll cells, halophytes can switch the CO₂ concentrating mechanism from the C₃ pathway to other pathways, such as C₄, crassulacean acid metabolism (CAM), and photorespiration. Photorespiration has been described as a positive regulation that functions as an energy sink when photoinhibition is triggered by salinity; however, photorespiration may hamper growth processes (Yamane et al., 2008). When exposed to high NaCl concentrations of 500 mM, halophytes such as *Salvadora persica*, *Arthrocnemum macrostachyum*, and *Sarcocornia fruticosa* have been found to have a higher rate of photorespiration and a slightly lower growth rate (Redondo-Gómez et al., 2006, 2010; Rangani et al., 2016; Pérez-Romero et al., 2020). Plants that fix CO₂ through the C₄ and CAM pathways, on the other hand, are said to be naturally better adapted to salt stress than C₃ plants (Stepien and Klobus, 2005; Vasquez et al., 2006). Under high salt concentrations, halophytes like *Puccinellia nuttalliana*, *Aeluropus littoralis*, and *Salicornia europaea* can move from C₃ to C₄ pathways, and halophytes like *Mesembryanthemum crystallinum* and *Portulacaria afra* can switch from C₃ to CAM pathways (Bose et al., 2017). Surprisingly, some halophytes, such as *Suaeda aralocaspica*, *Bienertia cycloptera*, and *Bienertia sinuspersici*, have both C₃ and C₄ pathways operating in the same cell, which is possible by the sorting of chloroplast that resemble mesophyll and bundle sheath cells (Caemmerer et al., 2014). Furthermore, roughly 30-45 percent of halophytes are C₄ plants, indicating that salinity favors the C₄ pathway over the C₃ pathway as a CO₂ concentrating mechanism (Feldman et al., 2008; Bromham and Bennett, 2014; Santos et al., 2016).

2.5.4 Photosynthesis under heavy metals

Heavy metal stress can affect chlorophyll and carotenoid biosynthesis negatively. Heavy metals can also result in changes in thylakoid membranes, affecting both structurally and functionally, thereby reducing PS II electron transport, decreasing the functionality of ferredoxin NADP⁺ reductase and plastoquinone synthesis (Huihui et al., 2020; Gill et al., 2022; Wang et al., 2022). This decreases the maximum electron transport capacity in the electron transport chain and reduces the photosynthetic efficiency of the plant (Anjum et al., 2016a,b). A study was conducted on the halophyte *Spartina patens* to analyze the effect of Cu stress on the photosynthetic efficiency of the plant. They found that high concentrations of Cu resulted in decreased electron transport rates (ETR) in chloroplasts, as they underwent photoinhibition at comparatively low photosynthetic active radiation (PAR) levels, thereby resulting in reduced production of chemical energy (Anjum et al., 2016b).

Higher Cu concentrations significantly decreased the chlorophyll content in *Spartina alterniflora* (Chai et al., 2014). This could be due to the inhibition of chlorophyll biosynthesis and the substitution of Mg ions of chlorophyll (La Fontaine et al., 2002; Boojar and Goodarzi, 2007; Yruela, 2009). Cambrolle et al. (2012) reported decreased chlorophyll content in the halophyte *Halimione portulacoides* exposed to Zn stress. Mature cucumber leaves under Cu stress exhibited decreased Photosynthesis (Vinit-Dunand et al., 2002). Cu damaged the chloroplast and thylakoid membranes of leaves of *Theobroma cacao* seedlings, affecting reduced photosynthetic efficiency (Souza et al., 2014). Increased Cu concentrations resulted in a decrease in the net photosynthetic activity of *Avicennia germinans* (Gonzalez-Mendoza et al., 2013). Ouzounidou and Ilias (2005) reported that the application of Cu reduced the net photosynthetic rate in *Helianthus annuus* plants, which was

caused by the closure of stomata, inactivation of Rubisco, and degradation of chlorophyll. Higher oxidative stress from 200 μM Cu resulted in decreased pigment composition and photosynthetic performance in *Lolium perenne* (Dong et al., 2014). *Lemna minor*, an aquatic angiosperm, exhibited reduced photosynthetic performance under Cu stress (Singh et al., 2022). The reduced photosynthetic performance under copper stress is mainly due to the Cu induced oxidative stress, thereby causing damage to PS II. The chlorosis of leaves in response to Cu also reduces the pigment content and results in low photosynthetic performance (Kumar et al., 2021b).

2.5.5 ROS and anti-oxidation under salinity

Salinity stress induces the production of ROS such as superoxide radical ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\text{OH}\cdot$) that ultimately affect the integrity of cell membrane, DNA, photosynthetic pigments (Cai-Hong et al., 2005; Mushtaq et al., 2020; Sachdev et al., 2021). Ashraf et al. (2019b) reported that salt stress is one of the causes of oxidative stress by the augmented production of ROS, which act upon cellular membranes, proteins, lipids, nucleic acids, and pigment molecules, thereby causing severe damage to living organisms. Similar observations were made by Duhan et al. (2019) in which salinity and water logging, individually or in combination, induce the production of ROS or affect cellular metabolism. Panda et al. (2019) reported that nitric oxide and H_2O_2 were significantly higher in *Haloxylon salicornicum* plants under higher salinity, and they concluded that both act as signaling molecules for activating genes responsible for salinity tolerance. Lipid peroxidation was enhanced due to higher salinity in plants, including halophytes. Similarly, 450 mM of NaCl treatment in *K. candel* was found to damage membranes and showed higher MDA content (Wang et al., 2014).

Environmental stresses like salinity, drought, waterlogging, temperature extremes, and high light intensity can induce ROS production in plants, affecting plant growth (Mittova et al., 2002). Salinity stress in plants leads to the significant accumulation of ROS, activating enzymatic and non-enzymatic antioxidants. In response to salinity, the protective antioxidant induction in plants imparts tolerance against oxidative stress (Garratt et al., 2002). Stressed plants produce non-enzymatic antioxidants such as phenolics, flavonoids, tocopherols, ascorbate (AsA), and glutathione (GSH), and enzymatic antioxidants such as superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), as well as the enzymes of the ascorbate glutathione cycle [GSH reductase (GR), AsA peroxidase (APX), mono-dehydroascorbate dehydrogenase (MDHAR), and dehydroascorbate reductase (DHAR)] to mitigate the effect of ROS (Chawla et al., 2013; Rakhmankulova et al., 2015; Soundararajan et al., 2019). Superoxide dismutase is a significant enzyme to scavenge superoxide anions from the plant cell. According to the metal ion bound to the enzyme, SOD is of three major types: manganese SOD (Mn-SOD), iron SOD (Fe-SOD), and copper/zinc SOD (Cu/Zn-SOD), and this enzyme detoxifies the toxic superoxide into hydrogen peroxide. Catalases and peroxidases get over-activated due to stress detoxifying the toxic levels of hydrogen peroxide (Wang et al., 2016a).

The halophytic plant *Cakile maritima* showed adequate tolerance under saline conditions by successfully activating its antioxidant system, including SOD, peroxidase, and enzymes of the ascorbate-glutathione cycle (Amor et al., 2019). The salinity tolerance of the seeds of halophytic plants *Arthrocnemum macrostachyum* (C₃ perennial) and *A. indicum* (C₄ perennial) was studied by Nisar et al. (2019) and they concluded that the germinating seeds of *A. macrostachyum* showed significant tolerance against salinity than *A. indicum* by the activation of CAT, ascorbate peroxidase (APX) and SOD.

Palliyath and Puthur (2018) reported that the halophytic plant *Bruguiera cylindrica* showed tolerance potential against high concentrations of NaCl (600 mM) by enhanced activation of enzymatic antioxidants such as APX, GPX, SOD, and CAT. Cai-Hong et al. (2005) reported that higher salinity induces the effective ROS scavenging mechanism in the C₃ halophyte *Suaeda salsa*, and it results in the reduction of ROS by upregulating antioxidant machinery, including ASA, GSH, GR, and APX.

2.5.6 ROS and anti-oxidation under heavy metals

Heavy metal stressed conditions result in the production of ROS in plants. Mitochondria, chloroplasts, and peroxisomes are the sites involved in the production of ROS. Heavy metal ions disturb chloroplast functions and decrease photosynthetic electron transport chain activity, leading to ROS production (Mittler et al., 2004). ROS production in mitochondria also occurs due to reduced electron transport chain activity due to the toxicity of heavy metal ions (Keunen et al., 2011). During photorespiration, hydrogen peroxide is generated in peroxisomes when glycolate is oxidized to glyoxylic acid (Mittler et al., 2004). Therefore, different types of ROS are produced due to the disturbance in the mitochondrial electron transport process (Sharma and Dietz, 2009). Cu stress was known to enhance hydrogen peroxide production in the plants *Trigonella foenum-graecum* and *Oryza sativa* (Thounaojam et al., 2012; Elleuch et al., 2013).

The destiny of ROS depends on the ROS scavenging system. When this system works appropriately, ROS functions as a signaling molecule. ROS can become toxic if the scavenging system does not function properly (Miller et al., 2010). Antioxidant defense systems in plants can act as ROS scavengers, thereby reducing the toxic effects of ROS, like oxidative damage (Qiu et al., 2008; Dazy et al., 2009; Kumari et al., 2010). Oxidative stress in plants can be eliminated by enzymatic and non-enzymatic antioxidants. The

antioxidant enzymes remove peroxides and other free radicals, reducing the deleterious effects caused by oxidative stress. Heavy metal stressed conditions activate most of the antioxidant enzymes in plants (Thounaojam et al., 2012). The activity of the antioxidant enzyme SOD, APX, and CAT increased under Cu stress in *Trigonella foenum-graecum* (Elleuch et al., 2013). Cu stress enhanced the activity of the enzymes SOD, CAT, and peroxidase in *Arabidopsis thaliana* (Drażkiewicz et al., 2004). The increased production of antioxidant enzymes has been reported in many halophytes under heavy metal stress. SOD activity increased in the halophyte *Acanthus ilicifolius* under Cd stress (Shackira and Puthur, 2017). An increase in the enzymes GPX and SOD activities was reported in *Sonneratia apetala* under Cd stress (Zhang et al., 2011). *Kandelia candel* and *Bruguiera gymnorrhiza* exhibited enhanced GPX activity under Cd stress (Zhang et al., 2007). Greater antioxidant enzyme activity was also reported in halophytes like *Kosteletzkya virginica*, *Salicornia brachiata*, and *Spartina alterniflora* by Rui-Ming et al. (2013), Sharma et al. (2010a,b) and Chai et al. (2014) respectively under heavy metal stress.

2.5.7 Osmotic regulation under salinity

Osmotic regulation is an essential physiological response of plants under salinity stress. It involves adaptive mechanisms that help to accumulate osmolytes and thus maintain the turgor pressure under salinity stress conditions (Singh et al., 2015). Osmoprotectants or osmolytes are low molecular weight compounds that help in the osmotic regulation of cells under stress. It protects cellular apparatus from dehydration but, at the same time, does not interfere with other metabolic processes. Various osmolytes are synthesized in plant cells in response to salt stress, such as ammonium compounds (polyamines, glycine betaine, α -alanine betaine, dimethyl-sulfonio propionate, and choline-O-sulphate), sugars and sugar alcohols

(fructan, trehalose, mannitol, D-ononitol, and sorbitol) and amino acids (proline and ectoine) (Singh et al., 2015). Besides the function of osmotic adjustment, osmoprotectants have the potential to detoxify ROS and thus reduce membrane damage and stabilize the enzymes and proteins (Ashraf and Foolad, 2007). According to Sadak (2019), irrigation with saline water induces the accumulation of osmoprotectants such as sucrose, trehalose, total soluble sugar (TSS), free amino acids, and proline in wheat than the control plants. Salt-induced P5CR, a crucial enzyme involved in proline biosynthesis, has been associated with high proline levels in transgenic plants, reducing cellular water loss and permeability of membranes, ultimately resulting in salt stress or osmotic stress tolerance (Kumar et al., 2019).

Polyamines are essential osmoprotectants that have the potential to reduce damage due to environmental stresses (Hussain et al., 2011). These are low molecular weight compounds that contain nitrogen and help in adjusting the pH of the cellular compounds (Groppa and Benavides, 2008). Ashraf and Foolad (2007) reported that osmolytes such as glycine betaine and proline are produced in plants in response to abiotic stresses, including salinity. It helps to protect biomembrane and macromolecules, scavenge ROS, and mediate osmotic adjustments (Annunziata et al., 2019). Sharma and Verslues (2010) reported that enhancement in proline accumulation occurs in plants under salinity stress, and later, it is broken-down during the recovery of the plants from stress. Proline has a role in osmo regulation, ROS scavenging, redox buffering, acting as molecular chaperones, and maintaining the integrity of membranes and proteins (Verbruggen and Hermans, 2008; Jisha and Puthur, 2014). Palliyath and Puthur (2018) reported that a significant accumulation of sugars occurs in the halophytic plant *Bruguiera cylindrica* in response to NaCl stress. The augmented level of

sugars in NaCl treated plants mainly contributes towards the build-up of the osmoticum of the cell sap.

2.5.8 Osmotic regulation under heavy metals

Plants accumulate osmotic solutes to counteract the changes in water potential, thereby stabilizing the structure and properties of macromolecules. This helps the plants to survive during stressed conditions, including heavy metal stress (Parida and Das, 2005; Li et al., 2010; Szafranska et al., 2011). Various osmolytes help plants tolerate heavy metal stress, including soluble sugar, free amino acids, and proline. Soluble sugar increases the tolerance of plants towards osmotic stress caused by heavy metal ions and offers protection from photo-damage (Rodriguez-Calcerrada et al., 2011). An enhancement in the accumulation of soluble sugars under heavy metal stress helps to maintain the structure of biomolecules and membranes (Irannejad and Shahbazian, 2004). Amino acids also aid in enhancing plants' heavy metal stress tolerance by improving phytochelatin production (Wu et al., 2013a).

Sruthi and Puthur (2019) found an increase in the soluble sugar content in the halophyte *Bruguiera cylindrica* in the presence of Cu in the growth medium. They reported that the relative water content percentage of the root and the total soluble sugar content were positively correlated. However, they also observed that proline and free amino acids had a better role in combating osmotic stress. They reported so because the increase in soluble sugar content was found only under lower concentrations of Cu. At higher Cu concentrations, the soluble sugar contents were found to decrease. At the same time, proline and free amino acid contents increased even under high concentrations of Cu (Sruthi and Puthur, 2019). A study by John et al. (2008) also reported that soluble sugar content increased at lower concentrations of heavy metals. However, soluble sugar content decreased

in the plant *Lemna polyrrhiza* at higher concentrations. An increase in soluble sugar and proline was found in *Suaeda maritima* grown heavy metal contaminated waste water (Saiyood et al., 2012).

Zygophyllum fabago, on exposure to heavy metal stress, accumulated more proline and soluble sugars (Lefevre et al., 2010a). The proline content in *B. cylindrica* also increased under Cu stress (Sruthi and Puthur, 2019). Shackira et al. (2017) found that proline, soluble sugars, and free amino acid accumulation in *Acanthus ilicifolius* enhanced under heavy metal stress. Proline neutralizes ROS, which in turn helps reduce damage caused by heavy metal stress (Szafranska et al., 2011). Proline ameliorates stress by playing multiple roles, such as metal binding, antioxidant defense, and signaling (Sharma and Dietz, 2006).

2.6 Ion homeostasis and ionomics

The ionome, an organism's mineral, nutritional, and trace element makeup, symbolizes the inorganic parts of cellular and biological systems. Ionomics is a crucial research area that aims to comprehend interactions between elements, between elements and genes, between elements and the environment, and the physiological and biochemical functions of each element. The ionomes are strictly under genetic control and differ amongst tissues (Singh et al., 2013). Minerals play vital roles in different plant functions. The whole mineral components are majorly classified into two: macro and micronutrients. The macro nutrients include C, H, O, N, P, K, Ca, S, and Mg, and the micronutrients include Zn, Mn, Fe, Cl, Cu, B, Mo, and Ni. Some elements are not strictly required for plants but can promote plant growth. These are classified under beneficial elements, including Al, Co, Na, Se, and Si.

Salinity in living habitats can limit plant growth and cause ion toxicity and an imbalance of nutrients. Severe salinity can disturb the distribution of each ion and can lead to metabolic disorders. Salt stress partially or fully prevents the uptake of N, P, K, Ca, Mg, and S and also affects the intake of trace elements like Fe, Cu, Zn, Mn, and B. Maintaining nutritional imbalance in the cells under saline stress can be achieved by regulating ion transport between organelles and maintaining ion homeostasis by imparting tolerance against salinity (Wu et al., 2013b; Kumari et al., 2015; Guo et al., 2019). In cotton plants, the NaCl stress caused an increase of Na content in all plant parts, but the K, Cu, B, and Mo content in roots and Mg and S in the leaves were reduced. The NaCl induced accumulation of ions like Cu, Mo, Si, P, and B in leaves of salt tolerant cotton was reported. The increase of these elements results from counteraction with increased Na in the tissue (Guo et al., 2019).

Under high NaCl conditions, Na ions enter the plant cell in various ways. The transport is majorly through selective and non-selective transporters, cation channels, etc. The increased Na ions can be excluded from the cytoplasm by compartmentalization and recirculation of Na ions out of the shoot by activating the Na^+/H^+ antiporter of the cell membrane. The higher level of Na ions in the plant cell causes membrane depolarization and, in turn, activates K^+ outwardly rectifying channels (KORs), resulting in the efflux of K ions from the plant cell. The reduced K ions and increased Na ions reduce the K^+/Na^+ ratio. The reduction of K ions is also due to the blockage of K specific channels, which occurs due to high Na in the plant cells. The ROS augmentation causes the activation of non-selective cation channels (NSCCs), and through this, K ions are also leaked out (Kumari et al., 2015).

There are reports of decreased Ca and Mg ions uptake and occasionally reduced carbon assimilation in halophytes with increasing Na ions concentration. However, extracellular Ca ions were significantly increased at the initial phase of NaCl stress. During salt stress, calcium is crucial for signaling. It is conceivable that extracellular Ca ions were found to directly alter Na ions influx first and then safeguard Na and K equilibrium through the SOS pathway (Mahajan et al., 2008). The saline tolerant *Sesuvium portulacastrum* maintains a high K and Ca level even under 600 mM NaCl and is instrumental in conferring salinity tolerance.

Interestingly, the content of Cu, Fe, Mn, and Zn were not affected in plants even during the high saline conditions (Venkatesalu et al., 1994). Even under severe NaCl stress, the halophyte *Aster tripolium* exhibits extraordinary K⁺ accumulation for maintaining ion homeostasis. Additionally, it has been found that the outer root area contains the largest concentrations of Ca, Fe, and Mg (Scheloske et al., 2004).

Heavy metal stress can alter plant parts' ion homeostasis and ionomics more critically than salinity. The Cd tolerant genotypes of maize plants exhibit functional uptake and translocation of the elements such as N, P, K, Ca, Mg, Zn, and Fe than sensitive varieties. The Cd in living habitats competes with Ca and replaces Cd due to its chemical similarity (Wu et al., 2021). The external application of Ca results in the reduction of Cd toxicity in plants, and this was reported in many plants such as *Cicer arietinum* (Sakouhi et al., 2022), *Nicotiana tabacum* (Wang et al., 2021), *Solanum lycopersicum* (Guo et al., 2018), *Brassica juncea* L. (Ahmad et al., 2015), pak choi seedlings (Huang et al., 2022), and *Macleaya cordata* (Cai et al., 2021). The alteration in the ionome of *Arabidopsis thaliana* subjected to Cu stress was reported by Lequeux et al. (2010). In the roots, Mg, Ca, Fe, and Zn increased, but at the same time, K and S were reduced. At the same time, in the shoot,

the content of K, Ca, P, and Mn decreased. The decrease of Ca in the shoot, coupled with an increase in the root, is another effect of Cu that has frequently been noticed in plants. Furthermore, it was reported that high Cu disrupts calcium transfer from the root to the shoot. The Mg, S, Fe, and Zn accumulation pattern in Cu stressed plant parts varies according to the physiological state and plant species (Panou-Filothou and Bosabalidis, 2004).

2.7 Metal binding molecules

Metal chelation is considered an essential heavy metal detoxification method in plants. It involves chelating toxic metals with organic acids, amino acids, peptides, and metalloproteins (Callahan et al., 2006). Phytochelatins (PCs) and metallothioneins (MTs) are considered an essential category of metal chelators. PCs and MTs are two classes of cysteine-rich, heavy metal-binding protein molecules (Cobbett and Goldsbrough, 2002). Among the metal binding molecules, these two are well studied. PCs and MTs are cysteine rich protein molecules, of which PCs are enzymatically synthesized, whereas MTs are gene encoded polypeptides (Cobbett and Goldsbrough, 2002).

2.7.1 Phytochelatins (PCs)

Heavy metals induce the synthesis of PCs; they are a thiol-rich family of proteins having a general structure (γ -Glu-Cys) n -Gly, where 'n' ranges from 2–5. Phytochelatins are derived from glutathione (GSH) and can chelate and detoxify the metals (Cobbett and Goldsbrough, 2002; Seregin and Kozhevnikova, 2023). The cysteine thiol groups of PCs help in the chelation process and form complexes with a molecular weight of 2.5–3.6 kDa (Sharma et al., 2022). The component amino acids of the metal binding PCs are cysteine, glycine, and glutamic acid. On exposure to heavy metals,

the plants start synthesizing PCs by activating the enzyme phytochelatin synthase, which acts upon a glutathione substrate to produce PCs. The synthesis of PCs takes place in the cytosol, and after complexing with metals, they are transported into the vacuoles. The rapid synthesis of PCs occurs in response to various heavy metals such as Cd, Cu, Zn, Ag, Au, Hg, and Pb (Zare et al., 2023). In the mangrove plant *Avicennia germinans*, overexpression of AvPCS (PC in *A. germinans*) occurs in response to high concentrations of Cu^{2+} and Cd^{2+} , which makes them highly tolerant to heavy metals (Tennstedt et al., 2009).

2.7.2 Metallothioneins (MTs)

Metallothioneins are another category of metal binding protein produced by the translation of MT mRNAs. They are low molecular weight, cysteine-rich, metal-binding proteins. The synthesis of these proteins gets enhanced in response to heavy metals. MTs have a dumbbell shaped conformation due to their two-metal binding cysteine-rich domains (Cobbett and Goldsbrough, 2002; Nordberg and Nordberg, 2022). MTs are classified into three, based on the arrangement of Cys residues. The class I MTs contain highly conserved 20 Cys residues based on mammalian MTs and are widespread in vertebrates. In contrast, in class II MTs, strict arrangements of cysteines are not present and are mainly seen in plants, fungi, and non-vertebrate animals (Kumar et al., 2022). Class III MTs differ markedly from class I and II MTs. They are enzymically derived and are most commonly composed of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n=2\text{--}11$). MTs are engaged in the cellular detoxification of heavy metals of Cd and Hg and in the homeostasis of Zn and Cu in animals and fungi. Gonzalez-Mendoza et al. (2007) reported the positive role of phytochelatins and metallothioneins in the metal (Cd and Cu) tolerance of black mangrove *A. germinans*. The enhanced synthesis of

these metal binding proteins provides detoxification potential to *A. germinans*.

2.8 Modulations in secondary metabolites under NaCl and CuSO₄ stress

Secondary metabolites are compounds that are not directly needed for the plant to function but play a vital role in the interaction of the plant with its environment to adapt and defend itself. The onset of stress triggers a broad range of cellular and molecular cross-talk and signaling cascades (Isah, 2019). Plant secondary metabolism and metabolites synthesized are the results of responses and adaptations of plants to various biotic and abiotic environmental stresses evolved in plants (Ali, 2014). Enhanced production of these compounds is a vital detoxification mechanism plants adopt to alleviate the detrimental effects of stresses. An increase in the synthesis of secondary metabolites like flavones, anthocyanins, phenolics, and certain phenolic acids may shield the cells from ion-induced oxidative stress by trapping these ions and decreasing their toxicity on cytoplasmic structures (Sytar et al., 2018).

The accumulation of secondary metabolites in response to abiotic stress conditions like heavy metals and salinity is a physiological trait linked to stress tolerance, and this can be advantageous for growing plants in contaminated areas. Expression of some related flavonoids genes (chalcone synthase, phenylalanine ammonia lyase, and flavonol synthase) and carotenoid genes (β -lycopene cyclase and phytoene synthase 2) were induced by NaCl in *Solanum nigrum* L., followed by enhanced production of lutein, β -carotene and quercetin 3- β -D-glucoside (Ben Abdallah et al., 2016). A remarkable increase in the concentration of flavonoids (22.5%–37.6%), tannic acid (15.1%–24.3%), and gossypol (26.8%–51.4%) was reported in cotton plants in response to salinity (Wang et al., 2015). Increased synthesis of total flavonoids and total saponins was observed in *Plantago ovata* with

the enhancement of NaCl in the medium (Zahra et al., 2012). *Rauvolfia tetraphylla* and *Catharanthus roseus* showed a considerable increase in reserpine and vincristine alkaloids when subjected to increased salt content in the growth media (Anitha and Kumari, 2006; Misra and Gupta, 2006). Similarly, salinity stress also increased the levels of phenolic compounds in lentils, wheat, and *Rosmarinus officinalis* (Ashraf et al., 2020; Kh et al., 2010).

Salvadora persica has improved salinity and As tolerance capability due to altered metabolite accumulation and exposure to salinity and As. Several phenolic molecules, including catechin, gallic acid, naringenin, quercetin, caffeic acid, rosmarinic acid, coumaric acid, and cinnamic acid, were upregulated in order to chelate metals and shield *S. persica* against As toxicity. Moreover, the concentration of polyphenols that increased considerably under high salinity were coumaric acid (+2.62-fold), gentisic acid (+2.47-fold), protocatechuic acid (+1.19-fold), cinnamic acid (+1.1-fold) and apigenin (+2.36-fold) (Patel and Parida, 2022).

A thorough comprehension of the mechanisms governing the synthesis, accumulation, and degradation of secondary metabolites would assist in formulating promising methods for improving secondary metabolite production and manipulating them to enhance plants' resilience to abiotic stresses. After Cu stress treatment, the up regulation of 85 genes associated with secondary metabolites was identified from grapevines. All of the genes were involved in the biosynthesis pathways of shikimic acid (9 transcripts), alkaloids (2), anthocyanins (45), lignin (21), and terpenoids (8). Upregulation of the genes involved in synthesizing lignins, stilbenes, and anthocyanins was also observed, demonstrating the enhancement of secondary metabolites frequently linked to enhanced plant resistance to Cu stress (Leng et al., 2015).

Increased activities of peroxidase (EC 1.11.1.7) and shikimate dehydrogenase (SKDH, EC 1.1.1.25) enzymes were observed in the hypocotyl of pepper (*Capsicum annuum* L.) plants growing in a nutrient media with excess copper (Díaz et al., 2001). Narula et al. (2005) observed that the presence of Cu stimulates diosgenin production in *Dioscorea bulbifera*. A similar result was observed in *Trigonella foenum-graecum* sprouts after exposure to different CuSO₄ concentrations. The highest level of diosgenin content was detected in the five mM CuSO₄ treatment, which was 41% higher than that in the control. In addition, it has been reported that Cu also enhances the betalains and artemisinin production in *Beta vulgaris* and *Artemisia annua*, respectively (Trejo-Tapia et al., 2001; Saghirzadeh et al., 2019).

2.9 Molecular adaptations in response to NaCl and CuSO₄

In several halophytes, various efforts have been carried out for genomic and transcriptomic analysis to identify salt-responsive genes, which were then functionally validated using transgenic methods. The majority of these genes code for proteins involved in signal transduction, antioxidant defense, and ROS scavenging, as well as Na⁺/H⁺ antiporters (plasma membrane or vacuolar), vacuolar pyrophosphatase, potassium transporters, ion channels, and antioxidants (Mishra and Tanna, 2017). Since plants have no known unique salt sensor or receptor, salinity-stress tolerance is a polygenic characteristic. The upregulation and activation of transcription factor genes such as WRKY, NAC, DREB, bZIP, and MYB happen upon sensing through ABA-dependent and independent pathways, giving the plants the ability to withstand salt stress (Chaudhry et al., 2021).

In the past ten years, numerous studies on the tolerance of plants towards salinity have focused on the potential halophytic species quinoa. Guard cells (GCs) were subjected to a transcriptome study in this plant. In

GCs, the transcript for the transporter AAP1, which is involved in absorbing neutral amino acids like proline, was substantially expressed. Salt stress exposure to quinoa significantly (340-fold) increased the sucrose transporter 2 (SUC2) gene, which may significantly affect stomatal motions. In response to salt stress, two genes (EFP1 and CHAL) were shown to be significantly elevated in quinoa. Both EFP1 and CHAL encode the secreted proteins that help prevent stomata's growth and differentiation. These expression level alterations in quinoa were associated with decreased stomatal density and may have something to do with improving the crop's water use effectiveness (Rasouli et al., 2023). In another study, salinity exposure to spinach was found to result in the overexpression of increased multiple genes associated with cell wall stiffness, including cellulose and lignin production and peroxidase, indicating that salt stress resulted in protective cell wall stiffening in this plant, imparting protection against the stress.

2.9.1 Phytochelatins synthase (PCS)

Phytochelatins (PCs) are thiol peptides that chelate heavy metals found in various plant species and some microbes. Phytochelatins synthase produces PCs non-translationally from glutathione (Cobbett, 2000). The post-translational activation of PCS, a constitutively expressed enzyme, is well known to occur in the presence of heavy metals. Studies using PCS mutants or overexpression provide abundant evidence for PCs' crucial role in heavy metal tolerance. Disruption of PCS resulted in increased hypersensitivity to heavy metals in *Caenorhabditis elegans* and *Schizosaccharomyces pombe* (Vatamaniuk et al., 2001). Various genetic experiments on PCS overexpression in various plant species have also demonstrated PCs' vital role in heavy metal detoxification or tolerance. Hetero-expression of the maize PCS gene *ZmPCS1* rescued the defective growth phenotypes of Cd sensitive *Arabidopsis atpcs1*. Additionally, *ZmPCS1*

overexpression in *Arabidopsis* improved Cd tolerance and accumulation. Higher glutathione and PC levels were the cause of this higher tolerance potential (Jin et al., 2022).

A. thaliana showed PC deficit and Zn and Cd hypersensitivity in the *cad1-3* defective mutant of AtPCS1. The metal hypersensitivity was alleviated by complementing *cad1-3* with several PCS, including TaPCS1, CePCS, and AtPCS1 (Heiss et al., 2003). In contrast to non-transgenic plants, *Ceratophyllum demersum* CdPCS1-expressing rice transgenic lines showed a notable increase in PCS activity and increased PC synthesis. The concentration of PCs, which also enhanced several-folds higher in transgenic lines, was positively linked with those lines' more excellent metal accumulation capability. The increased concentration of PCs in transgenic plants than in non-transgenic plants may have enabled the sequestration and detoxification of more As in the roots and shoots, limiting its accumulation in the grains (Shri et al., 2014).

Phytochelatin (PC) production was enhanced in roots of *Solanum nigrum* plants exposed to 200 $\mu\text{mol/L}$ Cu, contributing to a higher Cu accumulation in these organs, suggesting a role of PCs in the detoxification of this metal (Fidalgo et al., 2013). Cevher-Keskin et al. (2019) showed that upregulation of γ -EC and PCS was observed in *Brassica nigra* plants subjected to 200 and 500 μM Cu in the growth medium (Cevher-Keskin et al., 2019).

Although these results demonstrate that PCS is necessary for heavy metal stress tolerance, other studies have demonstrated that PCS also functions to control other abiotic stress responses in plants. Transgenic *Arabidopsis* and tobacco plants overexpressing AtPCS2 in high salt environments showed higher seed germination rates and seedling growth. Transcript levels of CuSOD2, FeSOD2, CAT2, and GR2 transcript levels were

significantly lower in wild-type *Arabidopsis*. Compared to wild-type plants, transgenic *Arabidopsis* exposed to salt stress had increased proline accumulation, decreased Na^+/K^+ ratios, lower hydrogen peroxide (H_2O_2) levels, and lipid peroxidation. These findings imply that AtPCS2 promotes seed germination and seedling growth under salt stress via several indirect actions that are probably connected to H_2O_2 scavenging, osmotic adjustment, and ion homeostasis (Kim et al., 2019).

2.9.2 Phenylalanine ammonia-lyase (PAL)

Heavy metals in the environment impact multiple levels of plant metabolism and activate various mechanisms that allow plants to fight both direct and indirect detrimental effects caused by the uptake and transport of metals within tissues. There is great interest in managing phenylpropanoid biosynthesis as a model for comprehending flux control in an intricate biosynthetic pathway and detecting targets for biotechnological manipulation of desirable products. The phenylpropanoid pathway is the main pathway for synthesizing numerous secondary metabolites, including phenolic compounds, under stress and physiological processes. A crucial link between primary and secondary metabolism, phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) facilitates the deamination of phenylalanine to generate cinnamic acid, the first step in the phenylpropanoid pathway (Huang et al., 2010). PAL is considered a crucial factor in forming phenols under metal stress. In general, soluble phenol accumulates more when PAL activity is higher. ROS is thought to mediate the communication between stress perception and PAL expression. In fern *Azolla imbricata* fronds treated with Cd, a positive association was observed between PAL activity and the formation of soluble phenolics and flavonoids (Dai et al., 2006). When sunflower roots were exposed to 50 mM CuSO_4 , enhanced activities of lignifying peroxidases, PAL, and antioxidant enzymes were detected. This

suggests that to reduce the effects of Cu-stress damage, sunflower roots activated several enzymatic defense mechanisms to ameliorate Cu-stress (Jouili and Ferjani, 2003). A similar increase in PAL activity at 25 and 50 μM CuSO_4 was reported by Ali et al. (2006) in *Panax ginseng*. After one day of treatment, Cu significantly increased lignin content by 71 and 148% and PAL activity by 5.4 and 12.1-fold in 60 and 120 μM CuSO_4 treatments, respectively, in *Matricaria Chamomilla* (Kováčik and Klejdus, 2008).

The accumulation of soluble phenols in the roots of *Hordeum vulgare* exposed to Co and Cu combined treatment was positively linked with the expression of the PAL-related gene (HvPAL). A tolerant variety of *Hordeum vulgare* (Yan66) had a more significant HvPAL transcription level, accompanied by a higher soluble phenol accumulation, conferring a high stress tolerance against heavy metals (Lwalaba et al., 2020). 2-Aminoindane-2-phosphonic acid (AIP) is an effective PAL inhibitor, having an essential role in the manipulation of phenolic biosynthesis. The effect of PAL inhibition by AIP in copper-exposed diploid and tetraploid chamomile roots has been studied by Kováčik et al. (2010). Suppression of root phenolic metabolism by AIP was sufficient, allowing Cu accumulation in diploid shoots. The AIP-induced rise in shoot Cu accumulation in diploid plants appears to be mediated by the reduction in phenol accumulation. These findings imply that these mechanisms are regulated at the gene expression level since diploid plants exhibited more PAL activity in Cu and Cu+AIP treatments than tetraploid ones.

Increased PAL activity in the radicles and hypocotyls of *Jatropha curcas* was found to be positively correlated with increasing NaCl concentrations (Gao et al., 2008). Osman and El-Naggar (2022) reported that the PAL gene activates the development of antioxidants, phenolic compounds, and tannins under stressful and salty conditions. Increased PAL

induction may be a protective response to cellular damage brought on by high salt levels. Salinity-stressed maize inbred lines showed transiently increased PAL gene expression that was similar to the antioxidant gene expression patterns in these plants, indicating that PAL may have a similar role in reducing the oxidative stress brought on by salinity (Gholizadeh and Kohnhrouz, 2010). *Aeluropus littoralis* (Gouan) parl, a halophyte grass, was studied to determine how varying NaCl concentrations (0, 200, 400 mM) affected growth, PAL gene expression as well as activity and lignin content at various internode sites (1st, 4th and 7th internodes). The results showed that NaCl stress reduces the development, elevates PAL mRNA levels, stimulates PAL activity, and triggers lignin deposition (Kelij et al., 2013).

3. MATERIALS AND METHODS

3.1 Plant Material

Volkameria inermis L. (earlier treated under the genus *Clerodendrum*) is an evergreen straggling shrub in the family of Lamiaceae and is categorized as a mangrove associate as well as a halophyte. The herbarium of the plant material was prepared, authenticated, and deposited in the Calicut University Herbarium (CALI), Department of Botany, University of Calicut, Kerala, India, with accession number 7054 (Fig. 1) (Sarath et al., 2024).

3.2 Experimental design

Healthy stem cuttings of *V. inermis* collected from natural habitats and cuttings were washed with tap water twice and then with a very mild concentration of detergent (teepol) to sterilize. Finally, the cuttings were washed with double distilled water and wiped using filter paper. These surface-sterilized stem cuttings were transferred to 15 μ M indole butyric acid (IBA) for 2 h at room temperature to induce root initiation. The IBA-treated stem cuttings were kept in paper cups (9×7 cm) filled with soil collected from the mangrove habitats for proper growth. After 80 days (80d) of growth, the rooted cuttings were transferred to 300 mL half-strength modified Hoagland medium in glass bottles (15×7 cm) after thoroughly washing the roots in tap water. Throughout the experiment, plantlets were kept in the polyhouse under controlled conditions at 60±2% relative humidity, 25±5°C temperature, and 12 h daylight (6 am to 6 pm) ranging from 28 to 600 μ mol m⁻² s⁻¹. The Hoagland nutrient solution was replenished twice weekly to avoid nutrient depletion and contamination by algal growth. The healthy plantlets of *V. inermis* were then subjected to varying concentrations of NaCl and CuSO₄ for 20d under hydroponic culture

conditions provided with a half-strength modified Hoagland medium. The leaves and roots were collected on 0, 5, 10, 15, and 20d of plant growth and used for various analyses. Plantlets grown in Hoagland's nutrient medium alone were designated as control, and those in the same medium containing various concentrations of NaCl and CuSO₄ as the treated plantlets (Sarath et al., 2024).

3.3 Composition and preparation of Hoagland nutrient solution

Modified Hoagland nutrient solution prepared according to the method of Epstein (1972) was used for the hydroponic culture studies. The stock solution of each nutrient was prepared separately just before use, from which an appropriate volume of each was mixed together to make up the final volume and concentration of the nutrient solution. pH of the solution was adjusted to 6.8 using 0.1 N HCl or NaOH (Table 1).

Table 1. Composition of modified Hoagland nutrient solution used in the study.

Compounds		Molecular weight	Concentration of stock solution		The volume of stock/L of the final solution
		g mol ⁻¹	mM	g L ⁻¹	mL
Macronutrients	KNO ₃	101.10	1000	101.0	6.0
	Ca(NO ₃) ₂ ·4H ₂ O	236.16	1000	236.16	4.0
	NH ₄ Cl	53.49	1000	53.49	2.0
	Mg(NO ₃) ₂ ·6H ₂ O	256.41	1000	256.41	1.0
Micronutrients	KCl	74.55	25	1.864	2.0
	H ₃ BO ₃	61.83	12.5	0.773	
	ZnSO ₄ ·7H ₂ O	287.54	1.0	0.288	
	MnSO ₄ ·H ₂ O	169.01	1.0	0.169	
	CuSO ₄ ·5H ₂ O	249.68	0.25	0.062	
	H ₂ MoO ₄	161.97	0.25	0.04	
	NaFeEDTA	558.50	53.7	30.0	0.3



Natural habitat of *V. inermis*



V. inermis at flowering period
(November-January)



Voucher specimen of *V. inermis*
deposited at CALI



V. inermis rooted plantlets transferred to Hoagland medium in glass bottles.
The Stress (NaCl and CuSO₄) was imparted at this stage

Figure 1. *V. Inermis*: Natural habitat and experimental condition

3.4 Determination of heavy metal concentrations in the soil samples of natural plant habitats and different plant parts of *V. inermis*

The soil and plant samples (*V. inermis*–leaves and roots) were collected from five different locations on the Arabian sea coastlines. The latitude and longitude of the sampling sites were noted (Table 2). The soil samples were the top soil collected from different locations in the study area. The top soil collected from a particular study area was mixed together to generate a single sample. Thus, the soil samples of five different study areas were prepared and labelled as S1 to S5. The leaves and roots of *V. inermis* were also collected from the five sample sites and washed with tap water to remove the dust particles. The samples- soil and plant parts were dried separately, and the dry weight was noted. The dried samples were transferred to the Kjeldahl flask, then concentrated nitric acid (69%) was added. The samples were heated in the heating mantle at 60°C and refluxed for digestion until the solution became colourless. After filtration through Whatman's filter paper, the digest was transferred to a standard flask and made up to 50 mL using distilled water. Heavy metals were quantitatively estimated using inductively coupled plasma-mass spectrometry (ICPMS) (Agilent 7800, Santa Clara, USA).

Table 2. Details of study sites selected for the present investigation

Location	Location Description	Latitude	Longitude
Site 1 (S1)	Kettungal	11°01'16"N	75°51'24"E
Site 2 (S2)	Ottummal	11°03'08"N	75°50'56"E
Site 3 (S3)	Alungal	11°04'16"N	75°50'58"E
Site 4 (S4)	Kadalundi Bridge	11°07'31"N	75°49'34"E
Site 5 (S5)	Balathiruthi	11°07'50"N	75°50'07"E

3.5 Screening to identify the concentration of NaCl and CuSO₄ at which plants exhibited the highest level of tolerance

The healthy plantlets of *V. inermis* were subjected to varying concentrations of NaCl (0, 100, 200, 300, 400, and 500 mM) and CuSO₄ (0, 100, 150, 200 and 250 µM) for 20d under hydroponic culture conditions provided with half-strength modified Hoagland medium. The leaves and roots were collected on 0, 5, 10, 15, and 20d of plant growth and used for various analysis. Plantlets grown in Hoagland's nutrient medium alone were designated as control, and those in the same medium containing various concentrations of NaCl and CuSO₄ as the treated plantlets. Screening analysis for identifying the effective concentration of NaCl and CuSO₄ at which the plants showed the highest tolerance level was done by analyzing various parameters such as total chlorophyll content, carotenoid content and lipid peroxidation in the plantlets on 20d of plant growth (Sarath et al., 2024).

3.6 Plant growth parameters

3.6.1 Fresh weight and dry weight

The plant samples from the plantlets were collected on 0, 5, 10, 15 and 20d. Before measuring the fresh weight, the roots and leaves were wiped with tissue paper to remove the dirt. After that, the weight of the samples was recorded using a weighing balance, which was considered as fresh weight. For dry weight determination, the samples were placed in an oven set at 100°C for 1h and followed by 60°C. The weight of the samples was noted on each day until a constant value was achieved.

3.6.2 Tissue water content

The plant samples from the plantlets were collected on 0, 5, 10, 15 and 20d and cleaned using tissue paper, and the same procedure for the

determination of fresh weight and dry weight was carried out. The tissue water content was measured by deducting the dry weight from the fresh weight.

Water content (g) = fresh weight – dry weight

3.6.3 Leaf area

The area of the leaves was measured by drawing the leaves outline on a piece of graph paper. The leaf area was then estimated by manually counting each division on the graph paper that was delineated by the borders of the leaves. To minimize experimental mistakes, mature leaves from the control and treated samples of comparable ages were randomly chosen for the measurements.

3.6.4 Tolerance index

Tolerance index (TI) percentage was calculated according to the protocol of Wilkins (1978).

$$TI(\%) = \frac{DW \text{ of treated plants}}{DW \text{ of control plants}} \times 100$$

Where DW is the dry weight of the leaves or roots of the plantlets.

3.7 Stress intensity factors

3.7.1 Superoxide ($O_2^{\bullet-}$) content

Superoxide content was estimated as described by Doke (1983).

Extraction: Pre-weighed plant samples were cut into 1×1 mm size and immersed in 5 mL of 0.01 M potassium phosphate buffer (pH 7.8) containing 0.05% NBT and 10 mM NaN_3 . The mixture was initially kept at room temperature for 1 h, later transferred to a water bath (85°C) and incubated

for 15 min. After incubation, the mixture was quickly transferred to the ice bath to reduce the temperature.

Estimation: After cooling, the absorbance of the mixture was measured at 580 nm using a UV-VIS spectrophotometer (Systronics 2201, Ahmedabad, India). The calculation and determination of $O_2^{\bullet-}$ content in the plant samples were carried out using the extinction coefficient of $12.3 \text{ mM}^{-1}\text{cm}^{-1}$. The $O_2^{\bullet-}$ content in the plant samples was expressed in millimoles per gram fresh weight.

3.7. 2 Hydrogen peroxide (H_2O_2) content

Hydrogen peroxide content was estimated as described by Junglee et al. (2014).

Extraction: Using mortar and pestle, pre-weighed plant samples were homogenized in 5 mL of 0.1% ice cold trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C . The supernatant was collected and used for the estimation of H_2O_2 content.

Estimation: One millilitre of the supernatant was mixed with an equal volume of potassium phosphate buffer (pH 7), followed by 1 mL of 1 M potassium iodide. The absorbance of the mixture was measured at 390 nm using a UV-VIS spectrophotometer. Hydrogen peroxide was used as the standard. The H_2O_2 content in the plant samples was expressed in micrograms per gram of fresh weight.

3.7.3 Membrane stability index (MSI)

The Membrane Stability Index (MSI) was calculated according to the method suggested by Sairam et al. (1997). Samples of fresh tissue were pre-weighed, cut into segments of 10 mm^2 size and immersed inside 20 mL of distilled water placed inside the tubes in two different sets. While the first

set was kept at a temperature of 40°C for a period of 30 min (C₁), the second set (C₂) was kept for a period of 15 min inside a boiling water bath (pre-set temperature of 100°C) for 15 min. Multi-parameter PCSTestor (Eutech Instruments, Vernon Hills, USA) was used to determine the electrical conductivities, EC₁ and EC₂. The MSI was then determined using the expression,

$$MSI = [1 - (EC_1/EC_2)] \times 100$$

Where EC₁ and EC₂ were the respective electrical conductivities at 40°C and 100°C temperatures.

3.7.4 Electrolyte leakage (EL%)

Electrolyte leakage (EL%) was estimated as described by Lutts et al. (1996) with some modifications. Pre-weighed fresh tissue samples were cut into 10 mm² sized segments and placed in tubes containing 20 mL of distilled water. The samples were kept at 4°C for 24 h, then brought to room temperature and the solution was transferred to another tube (C₁). Further, the tissue was again immersed in 20 mL distilled water and autoclaved for 15 min at 120°C (C₂). After cooling to room temperature, the electrical conductivities of both the solutions (EC₁ and EC₂, respectively) were measured using a Multi-parameter PCSTestor. The EL% was calculated as,

$$EL\% = (EC_1/EC_2) \times 100$$

3.7.5 Lipid peroxidation

The rate of lipid peroxidation was assessed based on the malondialdehyde (MDA) content estimation. Estimation of MDA was done according to Heath and Packer (1968).

Extraction: Pre-weighed plant tissues were homogenized in 5 mL of 5% TCA using mortar and pestle., The homogenate was centrifuged at room

temperature at 12,000 rpm for 15 min. The supernatant was collected and used for the estimation of MDA.

Estimation: 2 mL of the supernatant was mixed with an equal volume of 0.5% of thiobarbituric acid (TBA) prepared in 20% TCA. The solution was heated in a water bath (95°C) for 24 min, cooled and then centrifuged at 3000 rpm for 2 min. The absorbance of the supernatant was measured at 532 and 600 nm using a UV-VIS spectrophotometer. By subtracting the absorbance value at 600 nm from the absorbance value of 532 nm, the non-specific turbidity was corrected. Then, the MDA content was calculated using its extinction coefficient of 155 mM⁻¹ cm⁻¹. The MDA content in the plant samples was expressed in micromoles per gram of fresh weight.

3.8 Photochemistry

3.8.1 Total chlorophyll and carotenoid contents

According to the methods of Arnon (1949) and Lichtenthaler and Wellburn (1983), the contents of carotenoids and total chlorophyll in leaves were estimated, respectively. Using a pre-chilled mortar and pestle, a 500 mg fresh leaf sample was crushed in 80% acetone after being weighed using an electronic balance. The upper layer was recovered after centrifuging at 5000 rpm for 10 min at 4°C. Re-extraction with 80% acetone and centrifugation was repeated until the pellet became colourless. Using a UV-VIS spectrophotometer, the absorbance at 663, 646, 750, and 470 nm was recorded. Using the following formula, the amount of total chlorophyll (Chl *a+b*) and carotenoids in the extract was estimated as micrograms of chlorophyll and carotenoids per g of fresh weight.

$$\text{Chlorophyll } a + b = \frac{20.12 (A_{646} - A_{750}) + 8.02 (A_{663} - A_{750})}{\text{Fresh weight of the sample}} \times \text{volume}$$

$$\text{Carotenoids} = \frac{1000 (A_{470}) + 3.27 (\text{Chl } a - \text{Chl } b)}{\text{Fresh weight of the sample} \times 229} \times \text{volume}$$

$$\text{Chlorophyll } a = \frac{12.69(A_{663} - A_{750}) - 2.69(A_{646} - A_{750})}{\text{Fresh weight of the sample}} \times \text{volume}$$

$$\text{Chlorophyll } b = \frac{22.9(A_{646} - A_{750}) - 4.68(A_{663} - A_{750})}{\text{Fresh weight of the sample}} \times \text{volume}$$

3.8.2 Chlorophyll stability index (CSI)

The chlorophyll stability index (MSI) was measured as per the equation of Sairam et al. (1997), and chlorophyll content in the samples was calculated according to Arnon (1949). CSI was then calculated with the following equation:

$$\text{CSI} = (C_1 / C_2) \times 100$$

Where C_1 and C_2 are the total chlorophyll content in the plantlets under stress and control respectively.

3.8.3 Photosystem (PS) I and II activities

Thylakoids from leaves were isolated, according to Puthur (2000). The photochemical activities of the isolated thylakoids were assayed polarographically with a Clark-type oxygen electrode (DW1/AD, Hansatech, Norflok, UK) which was connected to a digital control box (OXYG1, Hansatech) at 4°C. The light dependent O_2 uptake/evolution was measured by irradiating the sample with the saturating intensity of white light ($1800 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) provided by a 100W halogen lamp (LS2, Hansatech). The activities of PSI and PSII were expressed in terms of $\mu\text{mol of } O_2 \text{ consumed (PSI)/evolved (PSII) min}^{-1} \text{ mg}^{-1} \text{ chlorophyll}$.

Isolation of thylakoid membranes: 500 mg of fresh tissue was homogenized in 5 mL ice-cold isolation buffer (pH 7.8) containing 400 mM sucrose, 10 mM

NaCl, and 20 mM tricine with pre-chilled mortar and pestle. The homogenate was filtered through 4 layered cheese cloth and then centrifuged at 4°C for 6 min at 5000 rpm. After centrifugation, the supernatant was discarded, and the pellet was suspended in 500 µM suspension buffer (pH 7.5) containing 100 mM sucrose, 10 mM NaCl, 20 mM HEPES [N(2-hydroxyethyl)piperazine-N-(2-ethanesulphonic acid)], and 2 mM MgCl₂. This thylakoid suspension was stored at 4°C and used to analyse thylakoid electron transport activities.

Estimation of chlorophyll content of thylakoid membranes: Chlorophyll content of the thylakoid samples was estimated according to the method of Arnon (1949). 40 µL of the thylakoid suspension was added to the test tube containing 3 mL of 80% acetone (v/v). The contents of the tubes were mixed thoroughly using a vortex mixer, the homogenate was centrifuged at 5000 rpm for 5 min, and the supernatant was collected. The absorbance was measured at 646, 663 and 750 nm against the solvent blank (80% acetone). The total chlorophyll content of the thylakoids was calculated using the following equation:

$$\text{Total Chl} = 20.12 (A_{646} - A_{750}) + 8.02 (A_{663} - A_{750}) \times \text{Dilution factor}$$

Analysis of thylakoid electron transport activities: For the measurement of PSI activity, stock solutions of 500 mM DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), 10 mM DCPIP (2,6-dichlorophenolindophenol), 500 mM ascorbate, 5 mM MV (methyl viologen) and 1 M sodium azide (NaN₃) were prepared. The 2 mL reaction mixture containing 20 µL each of DCMU, DCPIP, ascorbate and MV, 10 µL NaN₃, and 40 µL thylakoid extract was prepared in suspension buffer (pH 7.5). PSI activity was measured by monitoring oxygen consumption using DCPIP as an artificial electron donor and MV as an exogenous electron acceptor.

Preparation of pBQ

Reagents: Hydroquinone, potassium dichromate ($K_2Cr_2O_7$), concentrated sulphuric acid (H_2SO_4).

Preparation: 0.5 g hydroquinone is dissolved in 5 ml distilled water by heating till the solution becomes clear. 1g of $K_2Cr_2O_7$ is dissolved in 10 ml distilled water and to this slowly add 1 ml concentrated H_2SO_4 and the whole set up was kept in ice water. To this add hydroquinone solution dropwise and shake constantly until yellow crystals come out, which is pBQ. Dry it in an oven and store in $-20^\circ C$ in an amber coloured bottle.

For the measurement of PSII activity, 2 mL reaction mixture containing 20 μL pBQ and 40 μL thylakoid extract was prepared in suspension buffer (pH 7.5). PSII activity was measured in terms of oxygen evolution by using p-benzoquinone (pBQ) as an artificial electron acceptor.

The light dependent electron transport in the thylakoid membrane was regulated in certain steps by artificial electron acceptors and donors so as to estimate the PSI and PSII activities based on the O_2 uptake/evolution from/into the medium. For measuring PSI activity, PSII activity was blocked initially by adding DCMU to the medium. Electron transport to PSI was maintained by artificial electron donors, ascorbate and DCPIP in the medium, where ascorbate acted as reductant by donating electrons to DCPIP, and further, the electrons supplied by DCPIP to plastocyanin were transferred to PSI. Electrons from PSI were bypassed to an artificial electron acceptor, MV, in the reaction mixture. MV reacts with oxygen molecules in the medium and produces H_2O_2 . The dissociation of H_2O_2 to form oxygen and H_2O by the action of catalase was arrested by NaN_3 added to the reaction mixture. So that this oxygen will not interfere with depleting oxygen level as a result of reaction with MV. Thus, the

oxygen consumption by the activity of PSI alone was measured by the oxygen electrode system.

To measure the PSII activity, artificial electron acceptor pBQ was added to the medium, and it has the potential to accept the electrons from plastoquinone. The transfer of electrons from plastoquinone to cytochrome was terminated because of the action of higher reducing potential of pBQ, so the activity of PSII alone was measured. The splitting of water for transferring electrons to PSII results in the evolution of oxygen molecules in the medium, measured by an oxygen electrode system.

3.8.4 Chlorophyll (Chl) *a* fluorescence analysis

Using a Plant Efficiency Analyzer (Handy PEA, Hansatech Ltd., Norfolk, UK), measurements of chlorophyll fluorescence were analyzed. Light exclusion clips, clipped on the leaf surface, imparted dark adaptation for 20 min. By projecting a continuous red light ($3000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, 650 nm) from an array of three light-emitting diodes focused on a 5 mm diameter of the leaf surface, fluorescence measurements were taken up to 1 s with a data acquisition rate of 10 μs for the first 2 ms and 1 ms thereafter. With the use of the Biolyzer programme (Bioenergetics Laboratory, University of Geneva, Switzerland), the data collected from the Handy PEA was evaluated (Strasser et al., 2004). The table given below includes representations of all the fluorescence parameters chosen for the research.

Table 3. Detailing of the different fluorescence parameters described in this study

Parameters	Description
Phases in fluorescence induction curve	
$O = F_0$	Minimal fluorescence/first step of chl <i>a</i> fluorescence transient
$J = F_j$	The intermediate step in the chl <i>a</i> fluorescence transient at 30ms
$I = F_i$	The intermediate step in the chl <i>a</i> fluorescence transient at 2 ms
$P = F_m$	Maximal fluorescence level/ final step of chl <i>a</i> fluorescence transient
OJ-phase	Represents the reduction of the acceptor side of PSII
JI-phase	Represents the reduction of the PQ (plastoquinone) pool
IP-phase	Represents the reduction of the acceptor side of PSI
Area	The area above the fluorescence induction curve
Tf(max)	Time taken to reach F_m
Other JIP parameters	
$F_v = F_m - F_0$	Maximal variable fluorescence
F_v/F_0	Maximum efficiency of water splitting complex
F_v/F_m	Capturing and conversion efficiency of harvested light energy or maximum quantum yield of PSII
N	The turn over number of Q_A indicates the number of times Q_A was reduced from 0 to Tf(max)
SFI(abs)	Structure-function-index or the vitality index of PSII
PI(abs)	Performance index of PSII on absorption basis
PI(total)	Performance index of electron flux to the final PSI electron acceptors
DF(abs)	The PSII-relative driving force on an absorption basis
Fluorescence yield parameters	
PHI(Po)	Maximum quantum yield of primary PSII photochemistry (at $t = 0$)
PHI(Eo)	Quantum yield (at $t = 0$) for electron transport from Q_A^- to plastoquinone
PSI(Eo)	Probability (at $t = 0$) that a trapped exciton moves an electron into the electron transport chain beyond Q_A
Phenomenological energy flux parameters	
ABS/CSm	Absorption of energy per excited cross-section (CS) approximated by F_m
TRo/CSm	Excitation energy flux trapped by PSII of a photosynthesizing sample cross-section (CS) approximated by F_m .
ETo/CSm	Electron flux transported by PSII of a photosynthesizing sample cross-section (CS) approximated by F_m .
Dlo/CSm	Heat dissipation of excitation energy by PSII of a photosynthesizing sample cross-section (CS) approximated by F_m .

3.8.5 Leaf gas exchange parameters

Leaf gas exchange parameters were analyzed using an LI-6400 portable photosynthesis system (infra-red gas analyzer, LI-COR, Lincoln, Nebraska, USA). Leaf surfaces were cleaned and dried using tissue paper before being enclosed in the leaf chamber for gas exchange measurements. All the measurements were recorded on fully expanded *V. inermis* leaves, and reading was recorded between 9:00–10:00 AM at room temperature and ambient CO₂ conditions. The internal light source in LI-6400 was set at an intensity of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to ensure a constant and uniform light across all measurements. The various leaf gas exchange parameters, such as net photosynthetic rate, A_{net} ($\mu\text{mol m}^{-2} \text{s}^{-1}$), stomatal conductance, g_s ($\mu\text{mol m}^{-2} \text{s}^{-1}$), transpiration rate, T_r ($\text{mmol m}^{-2} \text{s}^{-1}$) and water use efficiency (WUE) were measured in *V. inermis* leaves after exposure to NaCl and CuSO₄ on 20d.

3.9 Osmolality and osmolytes

3.9.1 Estimation of osmolality

The osmolality of the leaves and roots was determined using a vapour pressure osmometer (Wescor 5520, California, USA) by following the methodology of Hura et al. (2007). The osmometer chamber was calibrated using 100, 290 and 1000 mmol kg^{-1} standard solutions (Wescor, 5520, USA). The samples were collected, dipped in liquid nitrogen, and transferred to a deep freezer (-80°C) for 30 min. For estimation, samples were thawed at room temperature. The sap that emerged from the sample was promptly collected with a pipette (10 μL) and added to the paper disc (SS-033, Wescor, USA) in the chamber of the osmometer. The osmolality of the cell sap was quantified in mmol/kg .

3.9.2 Total soluble sugar content

Total soluble sugar content was estimated using the phenol-sulphuric acid method (Dubois et al., 1956).

Extraction: Pre-weighed plant samples were homogenized in 5 mL of 80% ethanol using a pre-chilled mortar and pestle. The homogenate was centrifuged at 10000 rpm for 10 min at 4°C, and the supernatant was collected. The pellet was re-extracted using 80% ethanol, and the supernatant was pooled and used to estimate the total soluble sugar content.

Estimation: From the supernatant, a known aliquot volume was pipetted out and made up to 2 mL using distilled water. To this, 1 mL of 5% (w/v) phenol was added and mixed well. Then, 5 mL of concentrated sulphuric acid was added to the tube and cooled. The optical density of the solution was read at 490 nm using a UV-VIS spectrophotometer. D-glucose was used as the standard. Total soluble sugar content was expressed in milligrams per gram of fresh weight.

3.9.3 Total soluble proteins

The total soluble protein content of the plant material was determined using the Bradford (1976) method.

Extraction: Using a pre-chilled glass mortar and pestle, a 500 mg leaf sample was homogenized in 5 mL phosphate buffer (pH 7). The protein precipitate of the sample was collected by centrifugation at 5,000 rpm for 10 minutes at 4°C.

Estimation: After centrifugation, 0.1 mL of aliquot was pipetted and made up to a volume of 1 mL with distilled water. 5 mL of Bradford reagent was added to each aliquot, and the mixture was thoroughly homogenized by using a vortex mixture. A UV-VIS spectrophotometer was used to measure

the optical density of the solution at 575 nm. The standard used was bovine serum albumin (BSA) fraction V powder. Total soluble protein content was expressed as mg of protein per gram of plant tissue dry weight.

3.9.4 Total free amino acids content

Total free amino acids content in the plant tissues was estimated according to the method of Moore and Stein (1948) using ninhydrin-hydrindantin reagent.

Extraction: Pre-weighed plant samples were homogenized in 5 mL of 80% ethanol using a pre-chilled mortar and pestle. The homogenate was centrifuged at 10000 rpm for 10 min at 4°C, and the supernatant was collected. The pellet was re-extracted using 80% ethanol, and the supernatant was pooled and used to estimate total free amino acids content.

Preparation of reagent solution: 2 g ninhydrin and 0.3 g hydrindantin were dissolved in 75 mL 2-methoxyethanol (methyl cellosolve) and stirred carefully to avoid air bubbles in the solution. To this solution, 25 mL of sodium acetate buffer (pH 5.5) was added (3:1 ratio). The resulting reagent solution was immediately transferred into a dark bottle.

Estimation: From the supernatant, a known volume of aliquot was pipetted out and made up to 1 mL using distilled water. To this, 1 mL of freshly prepared reagent solution was added and kept in a boiling water bath for 15 min. After cooling, 5 mL of diluent (n-propanol and distilled water in a 1:1 ratio) was added, mixed well and incubated at room temperature for 15 min. The absorbance was measured at 570 nm using a UV-VIS spectrophotometer. L-leucine was used as the standard. The free amino acids content in the plant samples was expressed in milligrams per gram of fresh weight.

3.9.5 Proline content

Proline content in the plant samples was estimated according to the method of Bates et al. (1973) using ninhydrin reagent.

Extraction: Pre-weighed fresh samples were homogenized in 5 mL of 3% (w/v) aqueous sulfosalicylic acid using pre-chilled mortar and pestle. The homogenate was centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was collected and used for the estimation of proline.

Preparation of ninhydrin reagent solution: Mixed 20 mL of 6 M orthophosphoric acid and 30 mL of glacial acetic acid. 1.25 g of ninhydrin (2.5% w/v) was dissolved in this acid mixture.

Estimation: To a known volume of supernatant, an equal volume of glacial acetic acid and ninhydrin reagent was added and mixed well. The mixture was heated in a boiling water bath for 1 h, and then the reaction was terminated by placing the tubes in an ice bath. After cooling, 4 mL of toluene was added to the reaction mixture and stirred well for 30 s using a vortex mixer. The upper chromophoric toluene layer was separated, and the optical density was measured at 520 nm using a UV-VIS spectrophotometer. L-proline was used as the standard. The proline content in the plant samples was expressed in milligrams per gram of fresh weight.

3.10 Free radical scavenging mechanisms

3.10.1 Non- enzymatic antioxidants

3.10.1.1 Ascorbate (AsA) content

The estimation of AsA content was carried out according to the method of Chen and Wang (2002) with some modifications.

Extraction: Using mortar and pestle, pre-weighed fresh plant samples were

homogenized in 5 mL of 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was collected and used for the estimation of AsA content.

Estimation: An aliquot of 0.5 mL of the supernatant was mixed well with 0.25 mL of 0.2 M sodium phosphate buffer (pH 7.4). To this mixture, 0.5 mL of 10% (w/v) TCA, 0.5 mL of 42% (v/v) phosphoric acid, 0.5 mL of 4% (w/v) bipyridyl (dissolved in 70% alcohol) and 0.25 mL of 3% (w/v) ferric chloride were added. The mixture was incubated at 40°C for 30 min. The absorbance was measured at 525 nm using a UV-VIS spectrophotometer. The pure form of AsA was used as the standard. The AsA contents in the plant samples were expressed in milligrams per gram of fresh weight.

3.10.1.2 Glutathione (GSH) content

The estimation of GSH content was carried out according to the method of Chen and Wang (2002) with some modifications.

Extraction: Pre-weighed fresh plant samples were homogenized in 5 mL of 5% (w/v) trichloroacetic acid (TCA) using mortar and pestle. The homogenate was centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was collected and used for the estimation of GSH content.

Estimation: An aliquot of 0.5 mL of the supernatant was mixed with 2.3 mL of 0.2 M sodium phosphate buffer (pH 6.8). To this mixture, 0.2 mL of 6 mM 5,5'-dithiobis-2-nitrobenzoic acid (DNTB/Ellman's reagent dissolved in 0.2 M sodium phosphate buffer, pH 6.8) was added. The mixture was incubated at 30°C for 5 min. The absorbance was measured at 412 nm using a UV-VIS spectrophotometer. Oxidized glutathione was used as the standard. The GSH contents in the plant samples were expressed in milligrams per gram of fresh weight.

3.10.1.3 Total phenolics content

The assessment of the total phenolic content was carried out utilising the Folin-phenol Ciocalteu's reagent in accordance with the technique suggested by Folin and Denis (1915).

Extraction: Fresh plant samples that had already been weighed were homogenized in a mortar and pestle with 5 mL of 80% ethanol. The homogenate was centrifuged for 10 minutes at 10000 rpm at 4°C. The supernatant was collected, and the pellet was re-extracted using 80% ethanol. Pooled supernatant was taken to estimate the total phenolic content.

Estimation: A known volume of an aliquot from the supernatant was pipetted out and diluted with distilled water to a volume of 3 mL. This was then mixed well with 0.5 mL of 1 N Folin-Ciocalteu reagent. 2 mL of 20% sodium carbonate was added after 3 minutes. The solution was thoroughly mixed before being left to develop its colour for one minute. A UV-VIS spectrophotometer was used to measure the solution's optical density at 650 nm. The reference used was catechol. The amount of total phenolics was measured in milligrams per gram of fresh weight.

3.10.1.4 Flavonoids content

The extraction and estimation of flavonoid content was carried out according to the method of Mirecki and Teramura (1984) with some modifications.

Extraction: Pre-weighed fresh plant samples were cut into 1×1 mm size and immersed in 5 ml of solvent containing methanol, hydrochloric acid and water in 79:1:20 ratio. The mixture was incubated at room temperature for 24 h. After centrifugation, the resulting supernatant was used for the estimation of flavonoid content.

Estimation: The optical density of the supernatant was read at 315 nm using a UV-VIS spectrophotometer. The flavonoid content was calculated using its molar extinction coefficient of $33 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed in millimoles per gram of fresh weight.

3.10.2 Enzymatic antioxidants

3.10.2.1 Extraction for enzymatic assay

The enzyme extraction for all antioxidant enzymes was carried out according to the method of Polle et al. (1994). Pre-weighed fresh plant samples were homogenized in 5 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA using a pre-chilled mortar and pestle. The homogenate was filtered through four layered muslin cloth, and the filtrate was centrifuged at 14000 rpm for 15 min at 4°C, and the supernatant was used for the enzyme assay.

3.10.2.2 Superoxide dismutase (SOD, EC 1.15.1.1)

An assay of SOD activity in the fresh plant samples was done as per the modified protocol of Giannopolitis and Ries (1977). The reaction mixture consisted of 0.1 mL of 1.5 M sodium carbonate, 0.3 mL of 130 mM methionine, 0.3 mL of 13 μM riboflavin, 0.3 mL of 10 μM EDTA, 0.3 mL of 0.63 mM nitrobluetetrazolium (NBT) and 0.1 mL enzyme extract, and the reaction mixture was made up to 3 mL using 100 mM potassium phosphate buffer (pH 7.0). Different assay systems were set, *viz.*, test samples, dark-control and light-control. Test tubes containing all the assay mixtures, including enzyme extract, illuminated under a fluorescent lamp for 30 min served as the test sample and the set of tubes placed in the dark served as the dark control. The assay mixture without enzyme extract was illuminated under a fluorescent lamp for 30 min served as light control. The mixture without NBT and enzyme extract served as the blank. The blue formazan

accumulation in different tubes was quantified using a spectrophotometer by recording the absorbance at 560 nm against the blank. One unit of SOD activity was defined as the amount of enzyme required to inhibit the photochemical reduction of NBT to blue formazan by 50%.

3.10.2.3 Guaiacol peroxidase (POD, EC 1.11.1.7)

POD activity in the fresh plant samples was measured according to the method of Gasper et al. (1975) with some modifications. The reaction mixture (3 mL) contained 2.858 mL of 100 mM potassium phosphate buffer (pH 7.0), 30 µL of 1% guaiacol, 12 µL of 30% (w/v) hydrogen peroxide and 100 µL of enzyme extract. The activity of POD was recorded by observing the increase in absorbance at 420 nm for 3 min at 30 s intervals using a UV-VIS spectrophotometer. One unit of POD activity was defined as the amount of enzyme required to oxidize 1 µmol of guaiacol per min.

3.10.2.4 Ascorbate peroxidase (APX, EC 1.11.1.11)

APX activity in the fresh plant samples was assayed by following the method of Nakano and Asada (1981). The reaction mixture (3 mL) contained 2.589 mL of 100 mM potassium phosphate buffer (pH 7.0), 15 µL of 0.5 mM ascorbate, 296 µL of 30% (w/v) hydrogen peroxide and 100 µL of enzyme extract. The activity of APX was recorded by observing the decrease in absorbance at 290 nm for 3 min at 30 s intervals using a UV-VIS spectrophotometer. One unit of APX activity was defined as the amount of enzyme required to oxidize 1 µmol of ascorbate per min.

The specific activity of each enzyme was calculated after determining soluble protein concentration in the enzyme extract, according to Bradford (1976).

$$\text{Specific activity} = \frac{\text{Enzyme activity in Units}}{\text{mg protein/mL enzyme extract}}$$

3.11 Bioaccumulation studies

3.11.1 Bioaccumulation and translocation of Cu and Na

The method of Nardi et al. (2009) was used to digest plant samples in order to determine the Cu and Na bioaccumulation. The control, NaCl and CuSO₄ treated plant samples were collected on 20d and dried in an oven set at 100°C for 1h, and then the oven was set at 60°C until the weight attained a constant value. Dry weight was noted, transferred to Kjeldahl flasks, and then concentrated nitric acid (69%) as was added. The samples were heated in the heating mantle at 60°C and refluxed for digestion until the solution became colourless. The digest was then transferred to a standard flask, after the filtration through Whatman's filter paper, and made up to 50 ml using distilled water. The quantitative estimation of Na and Cu was done by using inductively coupled plasma-mass spectrometry (ICPMS) (Agilent 7800, Santa Clara, USA). The bioaccumulation of Cu in the plant samples was expressed in micrograms per gram dry weight. The translocation factor (TF) of Cu in the leaves was calculated by using the following equation,

$$\text{Translocation factor (TF)} = \frac{\text{Concentration of Na/Cu in the leaves}}{\text{Concentration of Na/Cu in the roots}}$$

3.11.2 Subcellular distribution of Na, Cu, K and Ca

The subcellular distribution of Na, Cu, K, and Ca in the cell wall and cell wall debris (fraction 1), chloroplasts and cell nuclei (fraction 2), mitochondria (fraction 3), and ribosomes, vacuoles, and cytoplasm (fraction 4) were determined according to the protocol of Wang et al. (2016b). The plant samples were homogenized in a pre-chilled mortar and pestle with an extraction buffer comprising 250 mM sucrose, 1.0 mM dithioerythritol, and 50 mM Tris-HCl (pH 7.5). To separate fraction 1, the homogenate was centrifuged at 300 g for 2 min. For fraction 2, the supernatant of fraction 1

was centrifuged once more at 2000 g for 15 min. The supernatant collected from fraction 2 centrifugation was then centrifuged again at 10,000 g for 20 min to obtain fraction 3, and the final supernatant was fraction 4. The pellet of all the fractions except fraction 4 was used for elemental analysis. All the centrifugation was done at 4°C using an ultracentrifuge (Sorvall WX100 Plus, ThermoFisher Scientific, USA). The subcellular fractions were dried at 70°C and then digested with HNO₃. The quantitative estimation of all the elements was done with inductively coupled plasma-mass spectrometry (ICPMS).

3.12 Ionomics of different plant parts

The quantitative estimation of the ions (Na, K, Ca, Mg, Mn, Fe, Ni, Cu, Zn, Mo, Cr, Al, and Se) was measured by using inductively coupled plasma-mass spectrometry (ICPMS) (Agilent 7800). The samples (root, stem, and leaves) were harvested and dried. The samples were prepared for analysis using the protocol of Nardi et al. (2009). The prepared samples were analyzed for Na, K, Ca, Mg, Mn, Fe, Ni, Cu, Zn, Mo, Cr, Al, and Se using ICPMS.

3.13 C, H and N analysis

Carbon, hydrogen and nitrogen content were determined using an elemental analyzer (Flash 200, Thermo Fisher Scientific, USA). Around 2 mg of the powder samples were weighed in soft universal tin containers (O.D: 0.5 mm × H: 0.8 mm) and sealed tightly without an air gap. Samples were loaded into the combustion chamber via the auto sampler. After combustion, the resulting gases (NO₂, CO₂ and H₂O) were carried by running helium gas, then swept through a GC column that provided separation of the combustion gases, and finally, the gases were detected by a thermal conductivity detector (TCD). Total run time was around 12 min. Elemental

analysis was carried out in triplicate, and the average value was reported for each element. Sulfanilamide ($C_6H_8N_2O_2S$) was used as the standard.

3.14 Fourier Transform Infrared (FTIR) spectroscopic analysis

The leaves, stems, and roots of *V. inermis* grown under control and stressed conditions were collected and thoroughly washed with distilled water. The dried samples were grinded into a fine powder using a mechanical grinder. The powdered samples were made into pellets after mixing with dried potassium bromide (KBr). The pellets were positioned in the beam's path, and the solid-state spectra were assessed using an FTIR spectrometer (Jasco-4100, Shanghai, China) with a 400–4000 cm^{-1} range and a 2 cm^{-1} resolution. In order to examine the data, Origin Pro 8 was used.

3.15 Anatomical modifications

3.15.1 Histochemistry

Leaves, stems, and roots of control and stressed plants (NaCl and $CuSO_4$) were collected and fixed in FAA (100 mL FAA contains 10 mL formalin, 5 mL acetic acid, 50 mL ethanol and 35 mL distilled water). Safranin and toluidine blue were used to stain free-hand sections. A compound microscope was used to take the photomicrographs (LEICA DM2000 LED microscope attached to LEICA DMC4500 camera). Phloroglucinol-HCl test was used for examining the lignified cells in the control and treated plants. It was prepared by dissolving 1 mL of phloroglucinol in 100 mL of ethanol (Johansen, 1940; Tandon and Sharma, 2018).

3.15.2 Scanning Electron Microscopy (SEM)

A scanning electron microscope (SEM) was used to study the anatomical characters of *V. inermis* (leaves and roots). The cross sections

were fixed in 2.5% gluteraldehyde, prepared in 0.2 M sodium cacodylate buffer (pH 7.4). An ascending series of acetone was used for dehydration purposes. Dry samples were mounted to aluminium stubs with double-sided conducting carbon tapes and then coated with gold and palladium for 60 seconds using a sputter coater. Field Emission Scanning Electron Microscope (FESEM) (Gemini 300, Carl-Zeiss Jena, Germany) was used to take the photomicrographs.

3.16 High Resolution Liquid Chromatography-Mass Spectrometry (HR LC-MS) analysis

Q-Exactive Plus Biopharma - High-Resolution Orbitrap was used for the identification and separation of non-volatile components. Direct infusion mass with ESI & APCI (Positive and negative mode ionization) was involved. Its resolving power was up to 280,000. The maximum scan speed was 12 Hz. The RF-Lens ion source was used for increased sensitivity. Advanced active beam guide intelligent ion beam management was used for high-flux ion sources. Its mass range (m/z) was 50-8000 amu, and its resolution was 1,40,000 @ m/z 200.

3.17 Gene expression studies

Total RNA from the samples were extracted using RNAiso Plus (TaKaRa Bio, Shiga, Japan). The concentration of RNA was estimated using Qubit®.4.0 Fluorometer. 28 ng of RNA was used for the first strand cDNA synthesis using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit. 1 μ l of cDNA from each sample with 5 μ l 2X Real Time PCR smart mix and gene specific primers as per manufactures' instructions were used for PCR. Gene specific primers for phenylalanine ammonia lyase (PAL) enzyme and phytochelatase synthase (PCS) were designed using primer-3 software.

3.18 Statistical analysis

Duncan's multiple range test and one-way ANOVA were used to analyze the results statistically (SPSS software 20.0). The Pearson correlation coefficient was used to assess the importance of variations between various metrics (R software Version 4.2.2). The data are an average observation from three independent experiments.

3.19 Chemicals

Analytical reagent (AR) or guaranteed reagent (GR) chemicals were purchased from Himedia, Merck, SRL, Qualigens and Spectrochem companies. Bovine serum albumin (BSA), riboflavin, glutaraldehyde, methyl viologen, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), L-ascorbate, ascorbate oxidase and L-dehydroascorbic acid were purchased from Sigma-Aldrich Co., USA.

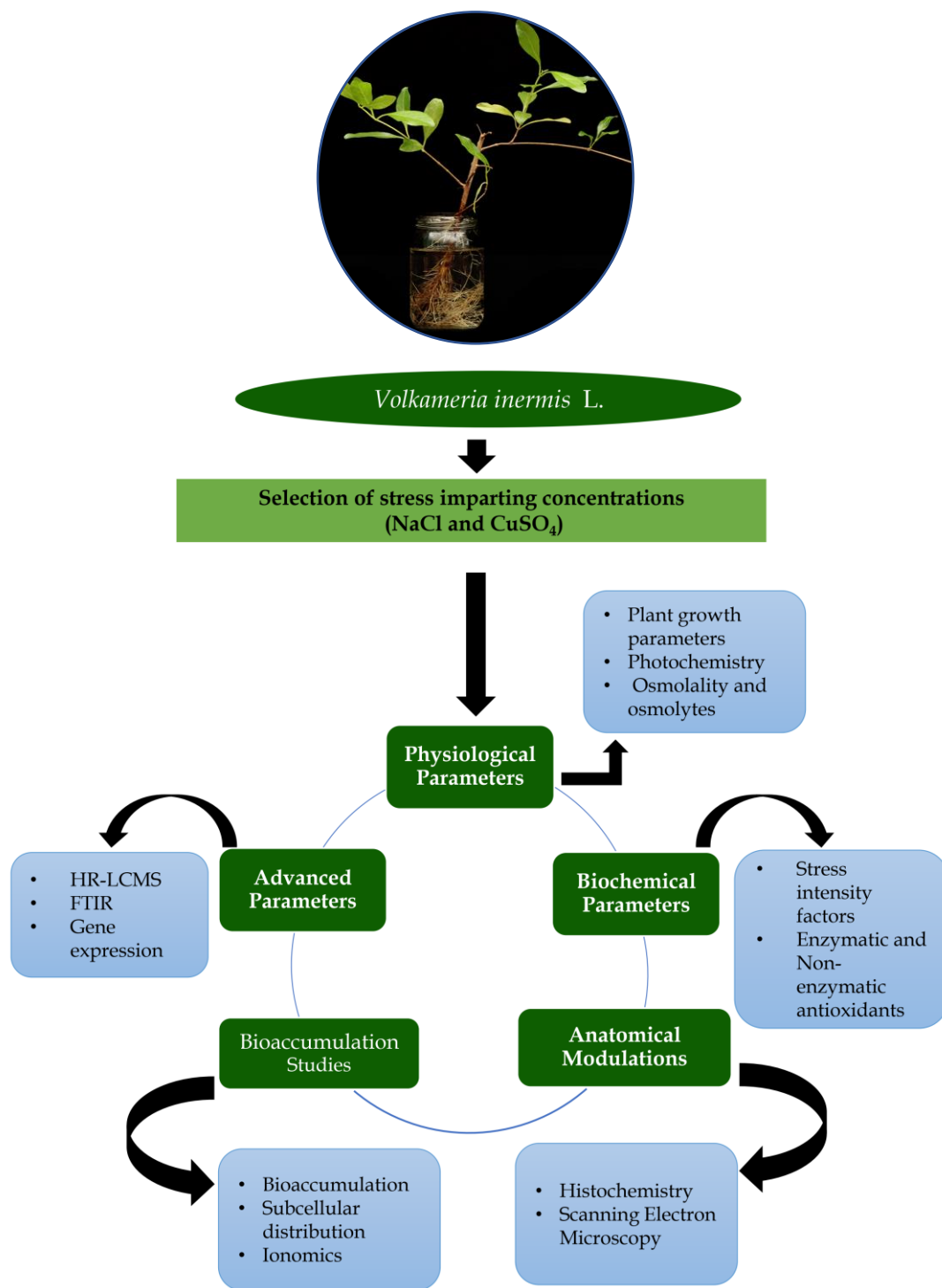


Figure 2. Schematic representation of the work

4. RESULTS

4.1 Heavy metal concentrations in the soil samples of natural plant habitats and different plant parts of *V. inermis*

For the present study, 5 polluted locations (mentioned in Table 2) were identified. The concentrations of the heavy metals Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Mo and Pb in the soil, leaves and roots of plants were evaluated and recorded (Table 4). The high concentrations of heavy metals in the soil points out the extent of heavy metal pollution in these sites. The range of concentrations of Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Mo and Pb ($\mu\text{g/g DW}$) in soil of different locations were 0.16-8.035, 20.84-136.89, 549.5-3462, 1.585-3.3, 5.125-24.5, 18.575-92.255, 0.11-5.185, 0.075-0.53, 0.115-0.81 and 1.335-40.96 respectively. The concentration of heavy metals in the soil followed the order $\text{Fe} > \text{Mn} > \text{Cu} > \text{Pb} > \text{Ni} > \text{Cr} > \text{Co} > \text{Zn} > \text{Mo} > \text{As}$. The leaves of plants collected from the five locations showed the concentrations of heavy metals Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Mo and Pb ($\mu\text{g/g DW}$) in the range of 1.42-6.94, 28.985-57.58, 30.085-86.91, 0.15-0.23, 1.41-4.18, 2.635-4.01, 0.02-1.235, 0.045-0.09, 0.595-2.45 and 0.88-1.495 respectively. The range of concentrations of Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Mo and Pb in the roots of plants in $\mu\text{g/g DW}$ were 5.95-30.61, 7.83-20, 4.88-119.43, 0.2-0.45, 0.06-1.81, 2.66-7.1, 0.22-2.51, 0.05-0.066, 0.01-0.43 and 1.03-10.23 respectively (Table 4).

4.2 Screening to identify the concentration of NaCl at which plants exhibited highest level of tolerance

The screening to identify the effective concentration of NaCl at which the plants showed the highest level of tolerance was carried out with 80d old healthy plantlets of *V. inermis*, which were subjected to varying concentrations of NaCl (0, 100, 200, 300, 400 and 500 mM) for 20d under

hydroponic culture conditions provided with half strength ($\frac{1}{2}$) Hoagland medium. Plantlets grown in $\frac{1}{2}$ strength Hoagland's nutrient medium was designated as control, and those in the same medium containing various concentrations of NaCl as the treated plantlets. To find out the tolerable concentration of NaCl, total chlorophyll, carotenoid and MDA content were analysed in the plantlets at an interval of 0, 5, 10, 15 and 20d of treatment.

The total chlorophyll content of the plantlets showed a decreasing trend upon increasing the concentration of NaCl and maximum reduction was observed in the plantlets treated with 500 mM of NaCl (70%) compared to the control plantlets. The NaCl treatment had less effects on chlorophyll biosynthesis up to 400 mM as compared to the control plantlets. The plantlets treated with 100 mM of NaCl showed an increase (12%) in chlorophyll content and there was no significant decrease in leaf total chlorophyll content of plantlets subjected to 200 and 300 mM NaCl as compared to control plantlets (Table 5).

The carotenoid content of the plantlets exhibited an increasing trend upto 400 mM NaCl compared with control plantlets. But the increase of the carotenoid content in 400 mM NaCl was negligible (6%) and it was lower than that recorded in other NaCl concentrations (100, 200 and 300 mM NaCl). A sharp increase in carotenoid content was observed in the plantlets treated with 100, 200 and 300 mM and it was 18, 30 and 59% respectively. The higher concentration of NaCl (500 mM) had detrimental effect on plantlets and carotenoid content was decreased by 13% compared with control plantlets. Moreover, the plants grown in 500 mM NaCl showed higher level of chlorosis and wilting compared with the control plantlets (Table 5).

The extent of lipid peroxidation in the control and NaCl treated plantlets were analysed based on the MDA content. The leaves of the

Table 4. Concentration of different heavy metals in the soil and root and leaf samples of *V. inermis* collected from the natural habitats.

Concentration of heavy metals ($\mu\text{g/g DW}$)											
Location	Sample	Cr	Mn	Fe	Co	Ni	Cu	Zn	As	Mo	Pb
S1	Soil	8.03 $\pm$ 0.40	68.89 $\pm$ 3.44	1005.83 $\pm$ 50.29	2.29 $\pm$ 0.11	5.12 $\pm$ 0.26	42.05 $\pm$ 2.1	0.11 $\pm$ 0.01	0.08 $\pm$ 0.004	0.41 $\pm$ 0.02	22.29 $\pm$ 1.11
	Leaf	1.92 $\pm$ 0.10	57.58 $\pm$ 2.88	36.67 $\pm$ 1.83	0.205 $\pm$ 0.01	3.4 $\pm$ 0.17	3.85 $\pm$ 0.19	0.875 $\pm$ 0.04	0.09 $\pm$ 0.005	2.45 $\pm$ 0.12	1.38 $\pm$ 0.07
	Root	30.61 $\pm$ 1.53	7.83 $\pm$ 0.39	4.88 $\pm$ 0.24	0.21 $\pm$ 0.01	0.196 $\pm$ 0.01	2.66 $\pm$ 0.13	0.22 $\pm$ 0.01	0.05 $\pm$ 0.002	0.25 $\pm$ 0.01	10.23 $\pm$ 0.51
S2	Soil	5.07 $\pm$ 0.25	136.89 $\pm$ 6.84	2119.08 $\pm$ 105.95	4.46 $\pm$ 0.22	13.3 $\pm$ 0.67	53.34 $\pm$ 2.67	5.18 $\pm$ 0.26	0.19 $\pm$ 0.01	0.57 $\pm$ 0.03	40.96 $\pm$ 2.05
	Leaf	1.42 $\pm$ 0.07	42.06 $\pm$ 2.10	60.03 $\pm$ 3.00	0.17 $\pm$ 0.01	4.18 $\pm$ 0.21	3.14 $\pm$ 0.16	1.23 $\pm$ 0.06	0.09 $\pm$ 0.004	1.38 $\pm$ 0.069	1.20 $\pm$ 0.06
	Root	30.1 $\pm$ 1.51	20 $\pm$ 1.00	55.76 $\pm$ 2.79	0.266 $\pm$ 0.01	1.81 $\pm$ 0.09	5.06 $\pm$ 0.25	2.33 $\pm$ 0.12	0.05 $\pm$ 0.002	0.016 $\pm$ 0.001	1.23 $\pm$ 0.06
S3	Soil	0.16 $\pm$ 0.01	96.93 $\pm$ 4.85	3462 $\pm$ 173.1	3.3 $\pm$ 0.17	24.5 $\pm$ 1.23	92.25 $\pm$ 4.61	2.79 $\pm$ 0.14	0.53 $\pm$ 0.03	0.81 $\pm$ 0.04	9.50 $\pm$ 0.48
	Leaf	3.99 $\pm$ 0.20	35.67 $\pm$ 1.78	81.47 $\pm$ 4.07	0.19 $\pm$ 0.01	2.05 $\pm$ 0.10	4.01 $\pm$ 0.20	0.415 $\pm$ 0.02	0.075 $\pm$ 0.003	1.01 $\pm$ 0.05	1.49 $\pm$ 0.07
	Root	13.38 $\pm$ 0.67	19.46 $\pm$ 0.97	18.26 $\pm$ 0.91	0.216 $\pm$ 0.01	1.11 $\pm$ 0.06	3.96 $\pm$ 0.19	2.01 $\pm$ 0.1	0.05 $\pm$ 0.002	0.433 $\pm$ 0.02	2.21 $\pm$ 0.11
S4	Soil	7.77 $\pm$ 0.39	20.84 $\pm$ 1.04	549.50 $\pm$ 27.48	1.58 $\pm$ 0.08	5.30 $\pm$ 0.27	18.57 $\pm$ 0.93	0.585 $\pm$ 0.03	0.075 $\pm$ 0.004	0.115 $\pm$ 0.006	1.33 $\pm$ 0.06
	Leaf	6.56 $\pm$ 0.33	28.98 $\pm$ 1.45	86.91 $\pm$ 4.35	0.23 $\pm$ 0.01	1.41 $\pm$ 0.07	4.31 $\pm$ 0.22	0.145 $\pm$ 0.01	0.045 $\pm$ 0.002	0.635 $\pm$ 0.03	1.10 $\pm$ 0.05
	Root	25.8 $\pm$ 1.29	12.93 $\pm$ 0.65	38.73 $\pm$ 1.94	0.21 $\pm$ 0.01	0.833 $\pm$ 0.04	4.93 $\pm$ 0.25	2.283 $\pm$ 0.11	0.05 $\pm$ 0.002	0.11 $\pm$ 0.005	1.43 $\pm$ 0.07
S5	Soil	6.36 $\pm$ 0.32	85.02 $\pm$ 4.25	1228.82 $\pm$ 61.44	2.62 $\pm$ 0.13	8.86 $\pm$ 0.44	38.06 $\pm$ 1.90	0.26 $\pm$ 0.01	0.16 $\pm$ 0.008	0.541 $\pm$ 0.002	3.21 $\pm$ 0.16
	Leaf	6.94 $\pm$ 0.35	41.74 $\pm$ 2.09	30.085 $\pm$ 1.50	0.15 $\pm$ 0.01	2.10 $\pm$ 0.11	2.63 $\pm$ 0.13	0.02 $\pm$ 0.001	0.045 $\pm$ 0.002	0.595 $\pm$ 0.02	0.88 $\pm$ 0.04
	Root	5.95 $\pm$ 0.29	10.18 $\pm$ 0.51	119.43 $\pm$ 5.97	0.45 $\pm$ 0.02	0.066 $\pm$ 0.003	7.10 $\pm$ 0.36	2.516 $\pm$ 0.13	0.066 $\pm$ 0.003	0.183 $\pm$ 0.01	1.033 $\pm$ 0.05

Table 5. Total chlorophyll, carotenoid and MDA content in *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300, 400 and 500 mM). Values are expressed as mean $\pm$ SE of three different experiments. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Parameters	NaCl Treatment (mM)	Days				
		0	5	10	15	20
Total chlorophyll content (mg/g FW)	0	0.828 $\pm$ 0.041 ^a	0.887 $\pm$ 0.044 ^b	0.829 $\pm$ 0.041 ^a	0.859 $\pm$ 0.042 ^b	0.816 $\pm$ 0.040 ^a
	100	1.05 $\pm$ 0.052 ^a	1.05 $\pm$ 0.052 ^a	0.895 $\pm$ 0.044 ^a	1.08 $\pm$ 0.054 ^a	0.910 $\pm$ 0.045 ^a
	200	0.956 $\pm$ 0.478 ^a	0.941 $\pm$ 0.047 ^b	0.853 $\pm$ 0.042 ^a	1.01 $\pm$ 0.050 ^a	0.730 $\pm$ 0.036 ^b
	300	0.815 $\pm$ 0.040 ^b	0.905 $\pm$ 0.045 ^b	0.721 $\pm$ 0.036 ^b	0.846 $\pm$ 0.042 ^b	0.674 $\pm$ 0.033 ^c
	400	0.712 $\pm$ 0.035 ^b	0.689 $\pm$ 0.034 ^c	0.643 $\pm$ 0.032 ^b	0.644 $\pm$ 0.032 ^c	0.505 $\pm$ 0.025 ^c
	500	0.709 $\pm$ 0.034 ^b	0.452 $\pm$ 0.022 ^d	0.411 $\pm$ 0.020 ^c	0.303 $\pm$ 0.015 ^d	0.252 $\pm$ 0.012 ^d
Carotenoid content (mg/g FW)	0	2.21 $\pm$ 0.712 ^c	2.66 $\pm$ 0.099 ^b	2.19 $\pm$ 0.162 ^b	1.35 $\pm$ 0.103 ^d	1.63 $\pm$ 0.628 ^c
	100	2.77 $\pm$ 0.130 ^a	2.45 $\pm$ 0.199 ^c	2.45 $\pm$ 0.022 ^a	2.94 $\pm$ 0.033 ^a	1.93 $\pm$ 0.960 ^c
	200	2.66 $\pm$ 0.155 ^a	2.09 $\pm$ 0.048 ^d	2.26 $\pm$ 0.175 ^b	2.68 $\pm$ 0.105 ^a	2.12 $\pm$ 0.088 ^b
	300	2.49 $\pm$ 0.164 ^b	2.84 $\pm$ 0.161 ^a	2.02 $\pm$ 0.144 ^c	2.58 $\pm$ 0.065 ^b	2.59 $\pm$ 0.127 ^a
	400	2.47 $\pm$ 0.143 ^b	2.66 $\pm$ 1.45 ^b	1.65 $\pm$ 0.226 ^d	1.79 $\pm$ 0.684 ^c	1.72 $\pm$ 0.047 ^b
	500	2.51 $\pm$ 0.125 ^b	2.58 $\pm$ 0.129 ^c	2.01 $\pm$ 0.101 ^c	1.45 $\pm$ 0.07 ^d	1.41 $\pm$ 0.070 ^d
Leaf MDA content (μ mol/g FW)	0	0.913 $\pm$ 0.045 ^e	0.806 $\pm$ 0.043 ^f	0.860 $\pm$ 0.043 ^f	1.02 $\pm$ 0.051 ^e	1.72 $\pm$ 0.086 ^f
	100	1.12 $\pm$ 0.056 ^d	1.34 $\pm$ 0.067 ^e	1.32 $\pm$ 0.66 ^e	1.98 $\pm$ 0.099 ^d	1.98 $\pm$ 0.099 ^e
	200	1.98 $\pm$ 0.99 ^c	2.01 $\pm$ 0.101 ^d	2.11 $\pm$ 0.105 ^d	2.56 $\pm$ 0.128 ^c	2.75 $\pm$ 0.137 ^d
	300	3.12 $\pm$ 0.156 ^b	3.56 $\pm$ 0.178 ^c	3.75 $\pm$ 0.187 ^c	3.75 $\pm$ 0.187 ^b	3.99 $\pm$ 0.199 ^c
	400	3.25 $\pm$ 0.162 ^b	3.75 $\pm$ 0.187 ^b	3.98 $\pm$ 0.199 ^b	3.99 $\pm$ 0.199 ^b	4.01 $\pm$ 0.201 ^b
	500	4.21 $\pm$ 0.210 ^a	4.28 $\pm$ 0.214 ^a	4.39 $\pm$ 0.219 ^a	4.85 $\pm$ 0.242 ^a	5.01 $\pm$ 0.250 ^a

Parameters	NaCl Treatment (mM)	Days				
		0	5	10	15	20
Root MDA content ($\mu\text{mol/g FW}$)	0	0.456 $\pm$ 0.022 ^f	0.403 $\pm$ 0.020 ^f	0.430 $\pm$ 0.301 ^f	0.510 $\pm$ 0.025 ^f	0.860 $\pm$ 0.043 ^f
	100	0.940 $\pm$ 0.047 ^e	1.85 $\pm$ 0.092 ^e	1.45 $\pm$ 0.072 ^e	1.77 $\pm$ 0.088 ^e	2.069 $\pm$ 0.103 ^e
	200	1.80 $\pm$ 0.090 ^d	2.12 $\pm$ 0.106 ^d	2.82 $\pm$ 0.141 ^d	2.95 $\pm$ 0.147 ^d	3.30 $\pm$ 0.165 ^d
	300	4.62 $\pm$ 0.231 ^c	4.89 $\pm$ 0.244 ^c	4.35 $\pm$ 0.217 ^c	3.17 $\pm$ 0.158 ^c	3.17 $\pm$ 0.158 ^c
	400	5.02 $\pm$ 0.251 ^b	5.43 $\pm$ 0.271 ^b	5.16 $\pm$ 0.258 ^b	4.70 $\pm$ 0.235 ^b	3.76 $\pm$ 0.188 ^b
	500	5.82 $\pm$ 0.291 ^a	5.87 $\pm$ 0.293 ^a	5.68 $\pm$ 0.284 ^a	5.98 $\pm$ 0.299 ^a	4.98 $\pm$ 0.249 ^a

plantlets treated with lower concentration (100 and 200 mM) of NaCl showed lower MDA content, but it was increased by 3-fold in plantlets treated with 500 mM NaCl. Similar trend of increase was also observed in the roots of plantlets treated with NaCl. The MDA content in roots was comparably higher than that in leaves and it was 6-fold higher in the roots of plantlets treated with 500 mM NaCl (Table 5).

The results of the preliminary phase of the study, which were based on the total chlorophyll, carotenoid and MDA content of the plantlets revealed that, *V. inermis* could effectively tolerate up to 400 mM, and signs of stress effects were shown in plantlets grown in concentration above it. As a result, for the remaining part of the investigation, *V. inermis* plantlets were only exposed to four concentrations of NaCl: 100, 200, 300, and 400 mM, along with control (Table 5).

4.3 Screening to identify the concentration of CuSO₄ at which plants exhibited highest level of tolerance

The screening to identify the effective concentration of CuSO₄ at which the plants showed the highest level of tolerance was carried out with 80d old healthy plantlets of *V. inermis*, which were subjected to varying concentrations of CuSO₄ (0, 100, 150, 200 and 250 µM) for 20d under hydroponic culture conditions provided with ½ strength Hoagland medium for 0, 5, 10, 15 and 20d of treatment. Plantlets grown in Hoagland's nutrient medium were designated as control, and those in the same medium containing various concentrations of CuSO₄ as the treated plantlets. To find out the tolerable concentration of CuSO₄, total chlorophyll, carotenoid and MDA content were analysed in the plantlets at an interval of 0, 5, 10, 15 and 20d of treatment.

The total chlorophyll content of the plantlets showed a decreasing trend upon increasing the concentration of CuSO₄ except 100 µM CuSO₄. The decrease was 14 and 22% in plantlets treated with 150 and 200 µM CuSO₄ respectively and the prominent reduction was observed in plantlets treated with 250 µM CuSO₄ (70%) compared to the control plantlets. The carotenoid content was increasing upon increasing the concentration of CuSO₄. There was a prominent and sharp increase observed upto 200 µM CuSO₄ and it was 1.6, 2 and 3-fold in plantlets treated with 100, 150 and 200 µM CuSO₄. The plantlets subjected to higher concentration of CuSO₄ (250 µM) exhibited an increase in carotenoid content but it was comparably less (18%). The MDA content in the leaves and roots of the plantlets subjected to CuSO₄ increased and reached its maximum in 250 µM CuSO₄ and the increase was 285% in leaves and 308% in roots as compared with control plantlets (Table 6).

Conclusively, the preliminary phase of the study imparting Cu stress to the plantlets of *V. inermis* by exposure to CuSO₄ revealed that, the plantlets were tolerant and had less negative effects upto 200 µM CuSO₄ as compared to the control plantlets. The prominent increase of MDA content in the leaves and roots of plantlets treated with higher concentration of CuSO₄ (250 µM) clearly imparted various detrimental effects. As a result, for the remaining portion of the investigation, *V. inermis* plantlets were only exposed to two doses of CuSO₄: 100 and 200 µM along with control.

4.4 Plant growth parameters

4.4.1 Fresh weight

The fresh weight of the leaves and root samples of the NaCl treated plantlets decreased progressively with increasing NaCl concentrations except the plantlets grown in 100 mM of NaCl. The reduction was 19, 50,

Table 6. Total chlorophyll, carotenoid and MDA content in *V. inermis* plantlets subjected to varying concentrations of CuSO₄ (0, 100, 150, 200 and 250 μ M). Values are expressed as mean $\pm$ SE of three different experiments. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Parameters	CuSO ₄ Treatment (μ M)	Days				
		0	5	10	15	20
Total chlorophyll content (mg/g FW)	0	1.59 $\pm$ 0.079 ^a	1.80 $\pm$ 0.094 ^a	1.616 $\pm$ 0.081 ^a	1.63 $\pm$ 0.035 ^a	1.20 $\pm$ 0.112 ^b
	100	1.41 $\pm$ 0.070 ^a	1.96 $\pm$ 0.098 ^a	1.618 $\pm$ 0.089 ^a	1.43 $\pm$ 0.133 ^a	1.40 $\pm$ 0.006 ^a
	150	1.57 $\pm$ 0.078 ^a	1.73 $\pm$ 0.086 ^a	1.02 $\pm$ 0.051 ^c	1.18 $\pm$ 0.048 ^b	1.03 $\pm$ 0.051 ^c
	200	1.80 $\pm$ 0.091 ^a	1.83 $\pm$ 0.091 ^a	1.18 $\pm$ 0.059 ^b	1.09 $\pm$ 0.138 ^b	0.938 $\pm$ 0.021 ^d
	250	1.22 $\pm$ 0.061 ^b	1.04 $\pm$ 0.052 ^b	0.744 $\pm$ 0.059 ^d	0.698 $\pm$ 0.013 ^c	0.357 $\pm$ 0.098 ^e
Carotenoid content (mg/g FW)	0	1.08 $\pm$ 0.065 ^b	1.84 $\pm$ 0.075 ^c	1.53 $\pm$ 0.266 ^c	1.77 $\pm$ 0.030 ^d	1.50 $\pm$ 0.259 ^e
	100	1.35 $\pm$ 0.154 ^a	1.42 $\pm$ 0.217 ^d	1.25 $\pm$ 0.263 ^d	1.33 $\pm$ 0.147 ^e	2.36 $\pm$ 0.066 ^c
	150	1.38 $\pm$ 0.039 ^a	2.21 $\pm$ 0.026 ^b	2.52 $\pm$ 0.237 ^b	2.65 $\pm$ 0.252 ^b	3.32 $\pm$ 0.166 ^b
	200	1.20 $\pm$ 0.061 ^a	2.37 $\pm$ 0.114 ^b	3.80 $\pm$ 0.190 ^a	4.86 $\pm$ 0.307 ^a	4.92 $\pm$ 0.384 ^a
	250	1.29 $\pm$ 0.135 ^a	2.53 $\pm$ 0.311 ^a	2.50 $\pm$ 0.208 ^b	1.97 $\pm$ 0.084 ^c	1.79 $\pm$ 0.101 ^d
Leaf MDA content (μmol/g FW)	0	0.806 $\pm$ 0.040 ^d	1.60 $\pm$ 0.081 ^e	1.22 $\pm$ 0.061 ^d	1.03 $\pm$ 0.051 ^e	1.73 $\pm$ 0.086 ^e
	100	0.967 $\pm$ 0.048 ^d	2.22 $\pm$ 0.111 ^d	3.01 $\pm$ 0.150 ^c	2.77 $\pm$ 0.138 ^d	3.40 $\pm$ 0.170 ^d
	150	2.05 $\pm$ 0.102 ^c	2.90 $\pm$ 0.145 ^c	3.43 $\pm$ 0.171 ^b	3.39 $\pm$ 0.169 ^c	3.92 $\pm$ 0.196 ^c
	200	3.06 $\pm$ 0.153 ^b	3.96 $\pm$ 0.198 ^b	3.61 $\pm$ 0.181 ^b	4.58 $\pm$ 0.229 ^b	4.71 $\pm$ 0.235 ^b
	250	4.33 $\pm$ 0.213 ^a	4.60 $\pm$ 0.230 ^a	5.27 $\pm$ 0.263 ^a	5.76 $\pm$ 0.288 ^a	6.67 $\pm$ 0.333 ^a
Root MDA content (μmol/g FW)	0	2.43 $\pm$ 0.158 ^d	3.79 $\pm$ 0.189 ^d	3.89 $\pm$ 0.194 ^e	3.48 $\pm$ 0.174 ^e	3.45 $\pm$ 0.172 ^e
	100	3.20 $\pm$ 0.128 ^c	3.54 $\pm$ 0.177 ^d	4.39 $\pm$ 0.219 ^d	4.67 $\pm$ 0.233 ^d	11.06 $\pm$ 0.553 ^d
	150	3.26 $\pm$ 0.420 ^c	4.19 $\pm$ 0.209 ^c	5.06 $\pm$ 0.253 ^c	8.75 $\pm$ 0.437 ^c	12.86 $\pm$ 0.643 ^c
	200	3.68 $\pm$ 0.479 ^b	5.18 $\pm$ 0.259 ^b	8.62 $\pm$ 0.431 ^b	12.72 $\pm$ 0.636 ^b	13.19 $\pm$ 0.659 ^b
	250	4.05 $\pm$ 0.783 ^a	6.14 $\pm$ 0.307 ^a	12.37 $\pm$ 0.618 ^a	13.83 $\pm$ 0.691 ^a	14.11 $\pm$ 0.705 ^a

52% in leaves and 37, 61, 71% in roots of *V. inermis* treated with 200, 300, and 400 mM NaCl respectively as compared to control plantlets on 20d. The fresh weight of leaves and roots of plantlets exposed to 100 mM NaCl increased slightly as compared to control plantlets (Table 7).

The treatment with CuSO₄ in *V. inermis* resulted in reduction of fresh weight. In the case of leaves, the reduction was severe in plantlets exposed to 200 µM CuSO₄ (63%) as compared with the control plantlets on 20d. The 100 µM CuSO₄ treatment did not affect the fresh weight except on 20d, where there was slight reduction (5%) as compared to the control plantlets. But in the case of roots, the fresh weight decreased progressively with respect to CuSO₄ concentration. The reduction was maximum in plantlets treated with 200 µM CuSO₄ as compared to the control plantlets. The decreasing trend was 20 and 53% in 100 and 200 µM CuSO₄ respectively as compared with control plantlets (Table 8).

4.4.2 Dry weight

The leaves and roots dry weight of the plantlets subjected to NaCl exhibited a reduction pattern except in the case of plantlets treated with 100 mM of NaCl. The reduction was maximum in plantlets treated with 400 mM of NaCl. In leaves, the reduction in dry weight was 45, 49, 59% in plantlets treated with 200, 300, and 400 mM NaCl respectively as compared to control plantlets. In the case of roots, the reduction was 39, 48 and 59% in plantlets treated with 200, 300, and 400 mM NaCl respectively as compared to control plantlets on 20d. There was an increase in dry weight of leaves and roots in plantlets treated with 100 mM of NaCl and the increase was 30 and 6% in leaves and roots respectively as compared with the control plantlets (Table 7).

In the case of plantlets treated with CuSO_4 , dry weight reduced upon increasing the concentration and it was maximum in the plantlets subjected to 200 μM CuSO_4 as compared with the control plantlets. The reduction in dry weight of the leaves was 29 and 58% in plantlets treated with 100 and 200 μM CuSO_4 as compared with the control plantlets on 20d. The severe effect of Cu stress was observed in the root dry weight. The reduction in root dry weight was 19 and 67% in plantlets treated with 100 and 200 μM CuSO_4 as compared with the control plantlets (Table 8).

4.4.3 Tissue water content

The tissue water status of the plantlets treated with NaCl showed a progressive reduction except in plantlets treated with 100 mM of NaCl. The reduction in tissue water content of the leaves was 9, 48, 49% in plantlets subjected to 200, 300 and 400 mM of NaCl respectively as compared with the control plantlets. In the case of roots, higher reduction was observed in 400 mM NaCl (73%) as compared with the control plantlets. The plantlets exposed to 200 and 300 mM NaCl exhibited 37 and 63% reduction in tissue water content as compared with the control plantlets. The lower concentration of NaCl (100 mM) imparted positive influence on tissue water content and the increase was 15 and 22% in leaves and roots respectively as compared with the control plantlets (Table 7).

The plantlets subjected to CuSO_4 , exhibited a reduction in tissue water content on 20d. The reduction was higher in roots than leaves. The leaves of the plantlets subjected to 200 μM CuSO_4 exhibited 51% reduction as compared with the control plantlets. In the case of roots, a progressive reduction in tissue water content was observed with increase in CuSO_4 concentration. The plantlets treated with 100 and 200 μM CuSO_4 recorded 9 and 55% reduction in tissue water content as compared with the control plantlets (Table 8).

Table 7. Fresh weight, dry weight and tissue water content of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300 and 400 mM). Values are the mean $\pm$ SE of three different experiments. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

Days	NaCl (mM)	Fresh weight (g)		Dry weight (g)		Tissue water content	
		Leaf	Root	Leaf	Root	Leaf	Root
0	0	6.45 $\pm$ 0.322 ^a	5.12 $\pm$ 0.256 ^b	1.98 $\pm$ 0.099 ^a	0.96 $\pm$ 0.048 ^b	4.47 $\pm$ 0.223 ^c	4.16 $\pm$ 0.208 ^b
	100	6.99 $\pm$ 0.349 ^a	5.78 $\pm$ 0.289 ^a	2.01 $\pm$ 0.101 ^a	1.08 $\pm$ 0.054 ^a	4.98 $\pm$ 0.249 ^b	4.70 $\pm$ 0.235 ^a
	200	5.91 $\pm$ 2.295 ^a	4.58 $\pm$ 0.229 ^c	0.98 $\pm$ 0.047 ^b	0.84 $\pm$ 0.042 ^c	4.97 $\pm$ 0.248 ^b	3.37 $\pm$ 0.168 ^c
	300	4.75 $\pm$ 0.237 ^b	4.01 $\pm$ 0.201 ^d	0.89 $\pm$ 0.044 ^c	0.78 $\pm$ 0.039 ^d	3.86 $\pm$ 0.193 ^d	3.23 $\pm$ 0.161 ^c
	400	6.89 $\pm$ 0.344 ^a	3.29 $\pm$ 0.164 ^e	0.93 $\pm$ 0.046 ^d	0.53 $\pm$ 0.026 ^e	5.96 $\pm$ 0.298 ^a	2.76 $\pm$ 0.138 ^d
5	0	6.71 $\pm$ 0.335 ^a	4.64 $\pm$ 0.232 ^b	1.95 $\pm$ 0.097 ^a	0.91 $\pm$ 0.045 ^a	4.76 $\pm$ 0.238 ^{ab}	3.73 $\pm$ 0.186 ^b
	100	7.12 $\pm$ 0.356 ^a	5.91 $\pm$ 0.295 ^a	1.98 $\pm$ 0.099 ^a	0.96 $\pm$ 0.048 ^a	5.14 $\pm$ 0.257 ^a	4.95 $\pm$ 0.247 ^a
	200	5.99 $\pm$ 0.299 ^b	4.26 $\pm$ 0.213 ^c	0.96 $\pm$ 0.043 ^b	0.82 $\pm$ 0.041 ^{ab}	5.12 $\pm$ 0.256 ^a	3.44 $\pm$ 0.172 ^c
	300	4.32 $\pm$ 0.216 ^c	3.93 $\pm$ 0.196 ^c	0.81 $\pm$ 0.040 ^c	0.71 $\pm$ 0.035 ^b	3.51 $\pm$ 0.175 ^b	3.22 $\pm$ 0.161 ^c
	400	4.21 $\pm$ 0.211 ^c	2.91 $\pm$ 0.145 ^d	0.64 $\pm$ 0.032 ^d	0.69 $\pm$ 0.034 ^b	3.57 $\pm$ 0.178 ^b	2.22 $\pm$ 0.111 ^d
10	0	6.56 $\pm$ 0.328 ^a	5.06 $\pm$ 0.226 ^b	1.99 $\pm$ 0.099 ^b	0.86 $\pm$ 0.043 ^a	4.57 $\pm$ 0.228 ^b	4.20 $\pm$ 0.211 ^b
	100	7.19 $\pm$ 0.359 ^a	5.98 $\pm$ 0.299 ^a	2.18 $\pm$ 0.106 ^a	0.91 $\pm$ 0.045 ^a	5.07 $\pm$ 0.253 ^a	5.07 $\pm$ 0.253 ^a
	200	5.31 $\pm$ 0.265 ^b	3.68 $\pm$ 0.184 ^c	1.16 $\pm$ 0.037 ^c	0.79 $\pm$ 0.039 ^{ab}	4.56 $\pm$ 0.228 ^b	2.89 $\pm$ 0.144 ^c
	300	3.85 $\pm$ 0.192 ^c	2.85 $\pm$ 0.142 ^d	0.97 $\pm$ 0.036 ^d	0.69 $\pm$ 0.034 ^b	3.12 $\pm$ 0.156 ^c	2.16 $\pm$ 0.108 ^d
	400	3.08 $\pm$ 0.154 ^d	2.21 $\pm$ 0.111 ^e	0.85 $\pm$ 0.030 ^e	0.65 $\pm$ 0.032 ^b	2.47 $\pm$ 0.123 ^d	1.56 $\pm$ 0.78 ^e

Days	NaCl (mM)	Fresh weight (g)		Dry weight (g)		Tissue water content	
		Leaf	Root	Leaf	Root	Leaf	Root
15	0	6.15±0.307 ^b	4.89±0.201 ^b	1.64±0.082 ^b	0.81±0.040 ^a	4.51±0.225 ^b	4.08±0.204 ^b
	100	7.26±0.363 ^a	5.75±0.287 ^a	2.13±0.103 ^a	0.83±0.041 ^a	5.20±0.26 ^a	4.92±0.246 ^a
	200	5.01±0.251 ^c	3.21±0.161 ^c	1.11±0.034 ^c	0.58±0.029 ^b	4.32±0.216 ^b	2.63±0.131 ^c
	300	3.68±0.184 ^d	2.12±0.106 ^d	0.91±0.031 ^d	0.51±0.025 ^c	3.05±0.152 ^c	1.61±0.081 ^d
	400	3.04±0.152 ^e	1.85±0.092 ^e	0.86±0.027 ^d	0.39±0.019 ^d	2.50±0.125 ^d	1.46±0.073 ^e
20	0	5.98±0.263 ^b	4.82±0.195 ^b	1.62±0.083 ^b	0.75±0.039 ^a	4.37±0.218 ^b	4.09±0.204 ^b
	100	7.12±0.356 ^a	5.71±0.285 ^a	2.11±0.101 ^a	0.80±0.035 ^a	5.09±0.254 ^a	5.00±0.251 ^a
	200	4.85±0.242 ^c	3.05±0.152 ^c	0.89±0.030 ^c	0.46±0.023 ^b	4.24±0.212 ^c	2.59±0.129 ^c
	300	2.98±0.144 ^d	1.86±0.093 ^d	0.83±0.029 ^c	0.39±0.017 ^c	2.40±0.121 ^d	1.51±0.075 ^d
	400	2.89±0.149 ^d	1.41±0.057 ^e	0.67±0.025 ^d	0.31±0.015 ^d	2.38±0.119 ^d	1.10±0.055 ^e

Table 8. Fresh weight, dry weight and tissue water content of *V. inermis* plantlets subjected to varying concentrations of CuSO₄ (0, 100 and 200 µM). Values are the mean ±SE of three different experiments. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

Days	CuSO ₄ Treatment (µM)	Fresh weight (g)		Dry weight (g)		Tissue water content	
		Leaf	Root	Leaf	Root	Leaf	Root
0	0	5.94±0.047 ^b	4.86±0.029 ^a	1.20±0.008 ^a	0.935±0.014 ^a	4.74±0.054 ^a	3.92±0.038 ^a
	100	6.09±0.073 ^a	4.27±0.032 ^c	1.08±0.053 ^b	0.945±0.027 ^a	4.98±0.092 ^a	3.34±0.026 ^c
	200	5.88±0.046 ^b	4.43±0.240 ^b	1.09±0.043 ^b	0.925±0.026 ^a	4.75±0.003 ^a	3.53±0.263 ^b
5	0	5.20±0.114 ^b	4.82±0.029 ^a	1.10±0.058 ^b	0.955±0.008 ^a	4.04±0.079 ^a	3.87±0.038 ^a
	100	5.34±0.053 ^a	4.28±0.017 ^b	1.25±0.026 ^a	0.905±0.026 ^b	4.07±0.037 ^a	3.39±0.033 ^b
	200	4.52±0.041 ^c	3.88±0.017 ^c	1.03±0.039 ^c	0.781±0.020 ^c	3.52±0.044 ^b	3.12±0.023 ^c
10	0	5.25±0.062 ^b	4.83±0.028 ^a	1.21±0.20 ^a	0.982±0.068 ^a	4.04±0.066 ^b	3.79±0.097 ^a
	100	5.37±0.075 ^a	4.33±0.032 ^b	0.91±0.039 ^b	0.738±0.035 ^b	4.44±0.113 ^a	3.57±0.010 ^b
	200	4.23±0.059 ^c	2.86±0.040 ^c	0.77±0.029 ^c	0.672±0.017 ^c	3.49±0.061 ^c	2.17±0.023 ^c
15	0	5.59±0.008 ^a	4.80±0.035 ^a	1.01±0.052 ^a	0.968±0.031 ^a	4.53±0.051 ^a	3.83±0.064 ^a
	100	5.36±0.079 ^b	3.91±0.035 ^b	0.89±0.046 ^b	0.774±0.029 ^b	4.47±0.116 ^b	3.12±0.026 ^b
	200	3.13±0.096 ^c	2.41±0.020 ^c	0.73±0.029 ^c	0.556±0.014 ^c	2.41±0.081 ^c	1.85±0.020 ^c
20	0	5.62±0.049 ^a	4.86±0.076 ^a	1.23±0.066 ^a	1.05±0.042 ^a	4.44±0.109 ^a	3.79±0.066 ^a
	100	5.36±0.029 ^b	3.93±0.024 ^b	0.875±0.023 ^b	0.851±0.023 ^b	4.51±0.052 ^a	3.09±0.012 ^b
	200	2.70±0.081 ^c	2.05±0.044 ^c	0.521±0.041 ^c	0.345±0.029 ^c	2.17±0.040 ^b	1.69±0.035 ^c

4.4.4 Leaf area

The leaf area of *V. inermis* on 20d of plant growth was reduced in response to 200, 300 and 400 mM of NaCl as compared to control plantlets. The higher reduction was found in the leaves of the plantlets treated with higher NaCl concentration (400 mM) and was 63% as compared with control plantlets. There was a positive influence of 100 mM NaCl on the leaf area of *V. inermis* and the rate of increase was 19% as compared to control plantlets (Table 9).

Similar to NaCl treatment, higher CuSO₄ treatment (200 µM) caused reduction in leaf area and the reduction was 45% as compared with control plantlets on 20d. The lower CuSO₄ treatment (100 µM) influenced positively on leaf area in plantlets as compared to the control plantlets (Table 9).

4.4.5 Tolerance index (TI)

The TI decreased upon increasing the concentration of NaCl. In leaves, TI was 55, 51 and 41% in plantlets treated with 200, 300 and 400 mM NaCl respectively as compared to control plantlets on 20d. Whereas the TI was significantly increased in the plantlets treated with lower NaCl concentration (100 mM) and it was above 100%. Similar observation was also found in the roots of the plantlets treated with NaCl. The TI was 61, 52 and 41% in plantlets treated with 200, 300 and 400 mM NaCl respectively as compared to control plantlets on 20d. An increase of 107% in TI was observed in the roots of the plantlets treated with 100 mM NaCl as compared with control plantlets (Table 9).

On exposure to CuSO₄, TI% was reduced in all the treatments on 20d. The TI calculated in leaves of plantlets treated with 100 and 200 µM CuSO₄ was 73 and 50% respectively as compared with the control plantlets. Similar trend of reduction was also observed in the case of roots. The TI was 80 and

54% in plantlets treated with 100 and 200 μM CuSO_4 respectively as compared with the control plantlets (Table 9).

4.5 Stress intensity Factors

4.5.1 Superoxide (O_2^-) content

There was a sharp increase in O_2^- content in plantlets treated with NaCl. The increase corresponds with the increase in the NaCl concentration. In leaves, NaCl induced O_2^- production was 91, 112, 117 and 154% in plantlets treated with 100, 200, 300 and 400 mM NaCl respectively as compared to control plantlets on 20d. The O_2^- content of the plantlets treated with lower NaCl concentration (100 and 200 mM) had almost lesser accumulation upto 10d, there after it increased drastically. In the case of roots, NaCl induced O_2^- production was significantly higher compared to leaves. The increase in O_2^- content was upto 9-13 fold in plantlets treated with 100, 200, 300 and 400 mM NaCl. The maximum augmentation in O_2^- content was found in the plantlets treated with 400 mM NaCl (13-fold) as compared to control plantlets (Fig. 3).

The plantlets treated with CuSO_4 recorded significant increase of O_2^- content in leaves and roots. In leaves, O_2^- content was increased to 2.6 and 4-fold in plantlets treated with 100 and 200 μM CuSO_4 respectively as compared with the control plantlets. Similar kind of increase was found in roots but it was more prominent than leaves. The increase was 6 and 15-fold in roots treated with 100 and 200 μM CuSO_4 respectively as compared with the control plantlets. The heavy metal induced O_2^- production on treatment with CuSO_4 was more prominent than NaCl treatment (Fig. 3).

4.5.2 Hydrogen peroxide (H_2O_2) content

There was a gradual augmentation of H_2O_2 content in leaves and roots of the plantlets subjected to NaCl. In leaves, NaCl induced H_2O_2

Table 9. Leaf area and Tolerance index (TI) of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300 and 400 mM) and CuSO₄ (0, 100 and 200 µM). Values are the mean ±SE of three different experiments. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

Concentrations	Leaf Area (cm²)	TI (%)	
		Leaf	Root
NaCl (mM) Treatment			
0	13.30±0.805 ^b	NA	NA
100	15.77±0.705 ^a	130±6.5 ^a	107±5.35 ^a
200	12.16±0.988 ^c	55±2.75 ^b	61±3.05 ^b
300	6.83±0.283 ^d	51±2.55 ^c	52±2.6 ^c
400	4.96±0.496 ^e	41±2.05 ^d	41±1.98 ^d
CuSO ₄ (µM) Treatment			
0	13.30±0.805 ^b	NA	NA
100	14.37±0.414 ^a	73±3.65 ^a	80±3.99 ^a
200	7.29±0.266 ^c	50±2.51 ^b	54±2.71 ^b

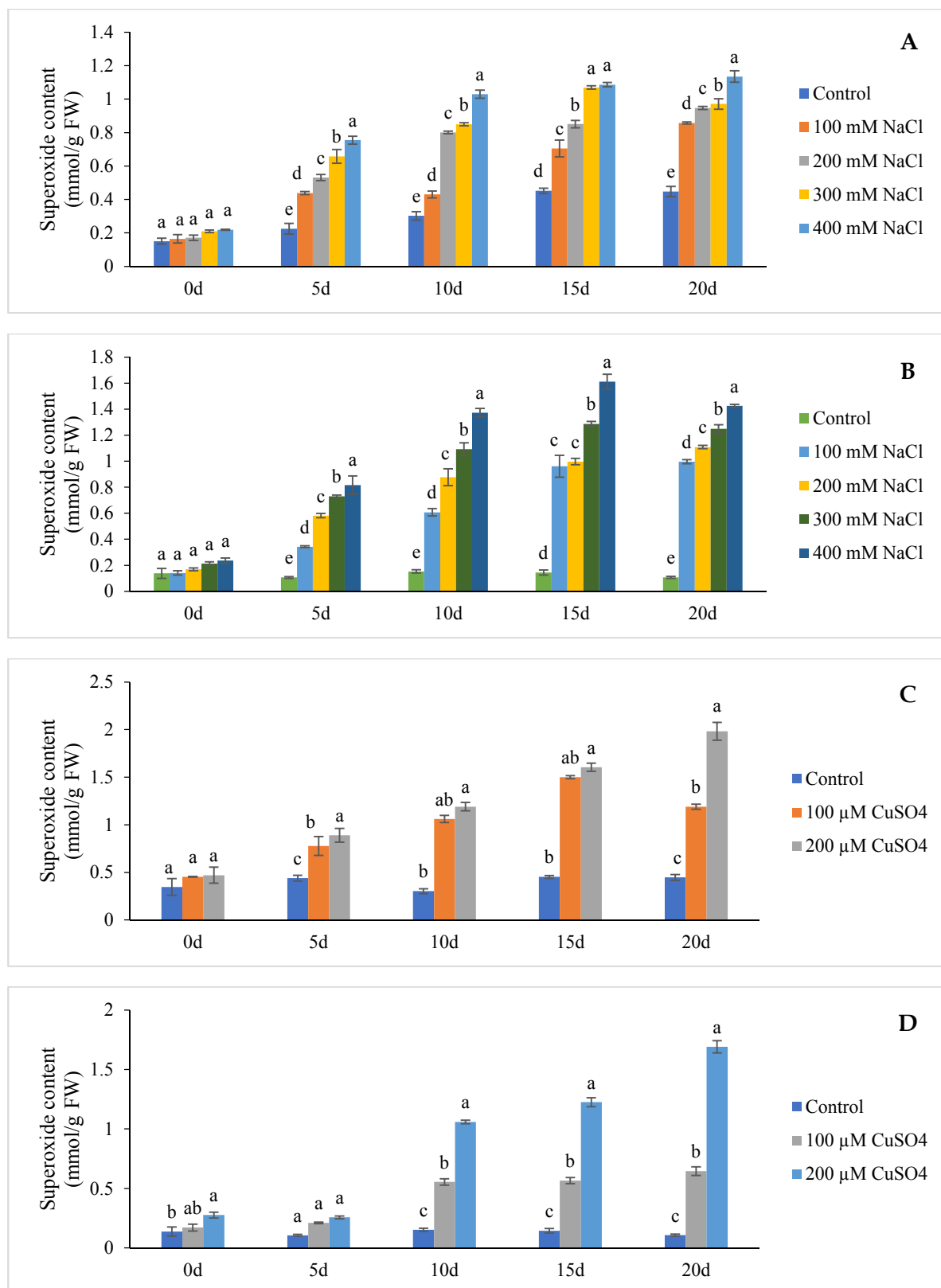


Figure 3. Superoxide content in leaves (A) and roots (B) of NaCl treated as well as leaves (C) and roots (D) of CuSO₄ treated *V. inermis* plantlets. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

production was more prominent from 10d onwards and exhibited its highest level on 20d. The increase in H_2O_2 content was 1.8-7.8-fold in plantlets treated with 100-400 mM NaCl as compared to control plantlets on 20d. similar observation was found in the root tissues on exposure to NaCl and it was 5.8-11.9-fold in plantlets treated with 100-400 mM NaCl as compared to control plantlets on 20d (Fig. 4).

CuSO_4 treatment caused an augmentation in the H_2O_2 content in leaves and roots tissues. The CuSO_4 induced H_2O_2 was 5 and 7-fold in leaves and 16 and 19-fold in roots on exposure of 100 and 200 μM CuSO_4 respectively as compared with the control plantlets. On exposure to CuSO_4 , root tissues accumulated significant amount of H_2O_2 compared with the leaves and also CuSO_4 treatments caused higher production of H_2O_2 than NaCl treatments (Fig. 4).

4.5.3 Membrane stability index (MSI)

Volkameria inermis plantlets on being subjected to higher NaCl concentrations, the stability of cell membranes was negatively affected as assessed by membrane stability index (MSI). In leaves, a significant reduction of MSI was recorded on 20d of exposing the plantlets to 400 mM NaCl concentration and the reduction was to the extend of 27% and 57% in leaves on treatment with 300 and 400 mM NaCl concentrations with respect to the control plants. Whereas, lower NaCl concentrations (100 and 200 mM NaCl) do not affect the MSI and interestingly it slightly improved the MSI. In roots, the NaCl treatment reduced the MSI, especially in 200 mM NaCl treatment. The lower concentration (100 mM) caused a negligible reduction in MSI as compared with the control plantlets. The extent of reduction in MSI was 51, 58 and 83% in plantlets treated with 200, 300 and 400 mM NaCl respectively as compared to control plantlets on 20d (Fig. 5).

On being subjected to CuSO₄, mild concentration (100 µM) the MSI did not alter and it was maintained as like that of control plantlets. The higher CuSO₄ (200 µM) treatment resulted in significant reduction of leaves MSI and the reduction was 74% as compared to control plantlets. In the case of roots, the extent of reduction was 59 and 83% on exposure to 100 and 200 µM CuSO₄ respectively as compared with the control plantlets (Fig. 5).

4.5.4 Electrolyte leakage (EL)

The exposure of plantlets to different concentrations of NaCl increased EL in leaves and root tissues. The increase of EL in the case of leaves of NaCl treated plantlets was 99, 201, 314% in 200, 300 and 400 mM NaCl treatments respectively as compared to control plantlets on 20d. The lower NaCl concentration (100 mM) did not show any increment; moreover, it exhibited a slight reduction of EL at all days of treatment as compared with control plantlets. In the case of roots, NaCl treatment resulted in gradual increase in EL and exhibited maximum EL in plantlets treated with 400 mM NaCl as compared to control plantlets on 20d. The increase in EL was 86, 248, 261 and 287% respectively in the roots of plantlets treated with 100, 200, 300 and 400 mM NaCl respectively as compared to control plantlets (Fig. 6).

On exposure to CuSO₄, leaves and roots exhibited a significant increase in EL%. The increase in EL% was 3.5 and 3.9-fold (in leaves) and 2.8 and 4.3-fold (in roots) on exposure to 100 and 200 µM CuSO₄ respectively as compared with the control plantlets. The higher increase in EL was observed in the plantlets treated with heavy metal (CuSO₄) than the NaCl treated plantlets (Fig. 6).

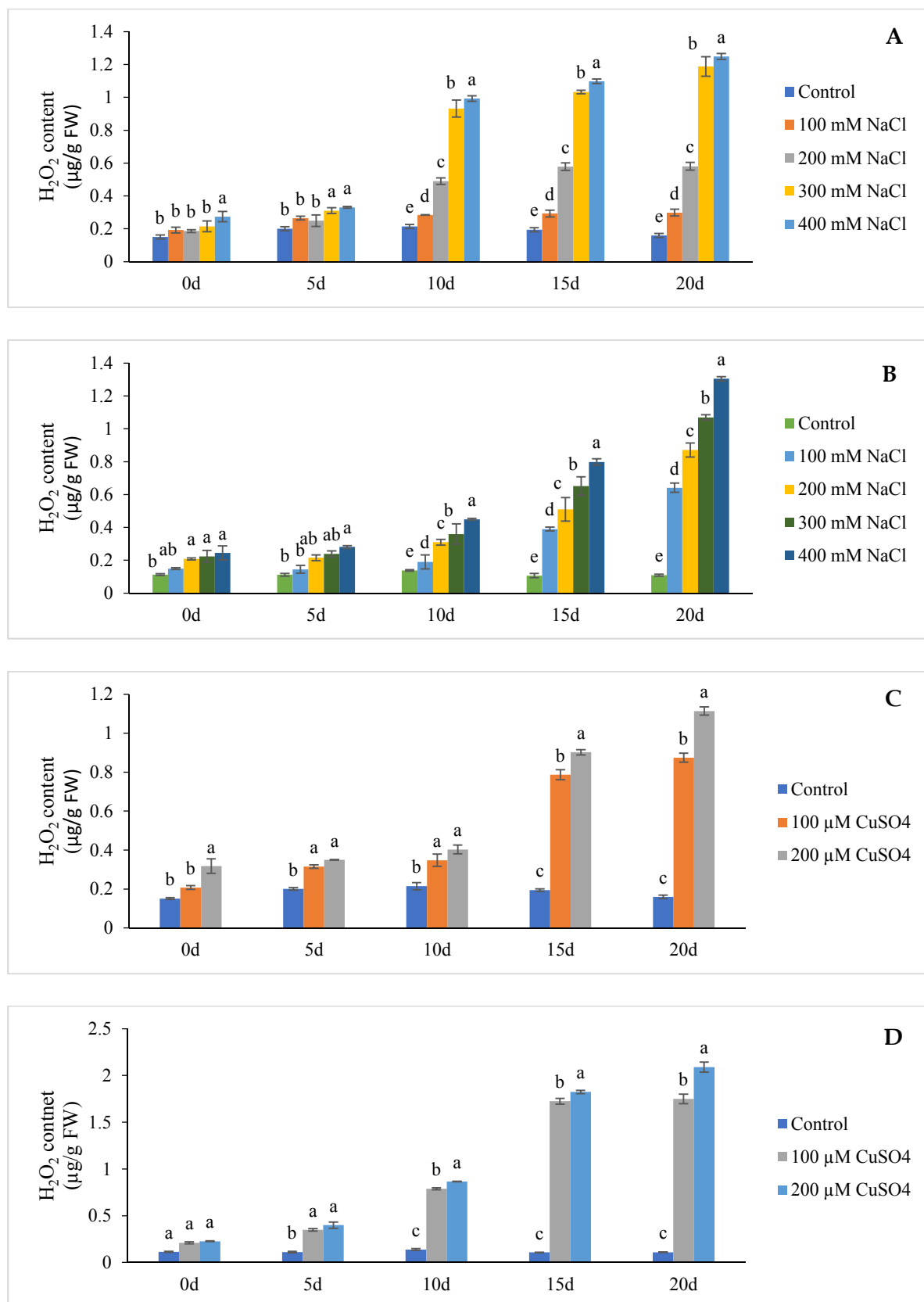


Figure 4. Hydrogen peroxide (H₂O₂) content in leaves (A) and roots (B) of NaCl treated as well as leaves (C) and roots (D) of CuSO₄ treated *V. inermis* plantlets. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

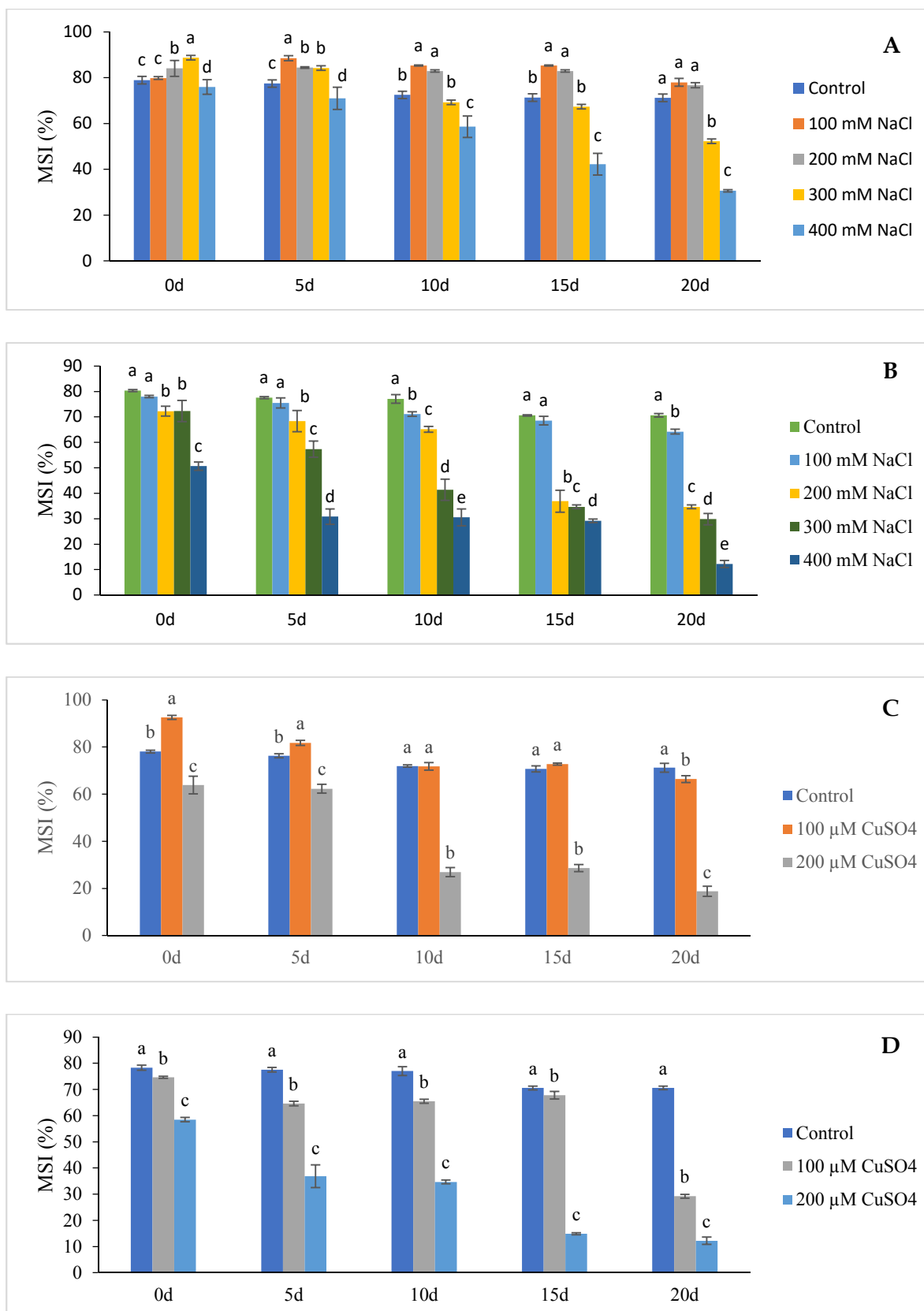


Figure 5. Membrane stability index (MSI) of NaCl treated leaves (A), roots (B) as well as CuSO₄ treated leaves (C), roots (D) of *V. inermis*. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

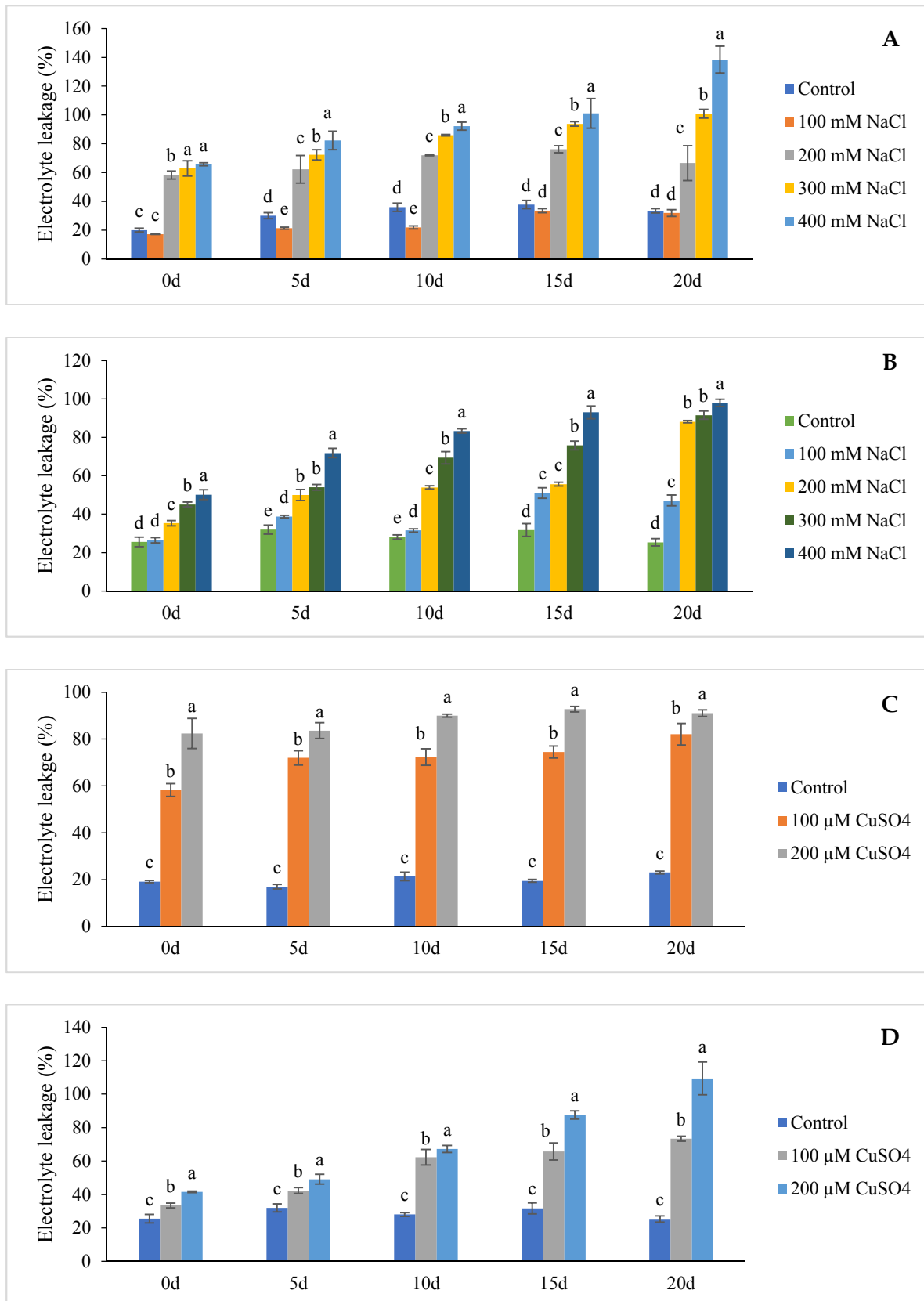


Figure 6. Electrolyte leakage in leaves (A) and roots (B) of NaCl treated as well as leaves (C) and roots (D) of CuSO₄ treated *V. inermis* plantlets. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

4.5.5 Lipid peroxidation

The lipid peroxidation of the control and NaCl treated plantlets were analysed based on the MDA content. The leaves of the plantlets treated with lower concentration (100 and 200 mM) of NaCl showed lower MDA content, but it was increased by 2.3-fold in plantlets treated with 400 mM NaCl as compared with the control plantlets. Similar trend of increase was also observed in the roots of plantlets treated with NaCl and the highest MDA content was found in the root tissues of the plantlets treated with 400 mM NaCl (4.3-fold) as compared with the control plantlets. The root MDA content was comparably higher than leaves in plantlets treated with NaCl (Fig. 7).

The MDA content of the leaves and roots of the plantlets subjected to CuSO₄ showed an increasing trend and reached its maximum in plantlets treated with 200 µM CuSO₄. The plantlets exposed to 100 and 200 µM CuSO₄ exhibited an increase of 96 and 172% (in leaves) and 220 and 281% (in roots) respectively as compared with control plantlets (Fig. 7).

4.6 Photosynthetic efficiency

The photosynthetic efficiency of *V. inermis* in response to NaCl and CuSO₄ were analysed by assessing parameters such as total chlorophyll and carotenoid content, photosystem activities, chlorophyll *a* fluorescence and leaf gas exchange parameters.

4.6.1 Total chlorophyll content

The total chlorophyll content in the leaves of the plantlets decreased with increasing the concentration of NaCl, except the plantlets subjected to 100 mM NaCl. The trend of increase in chlorophyll content of plantlets subjected to 100 mM NaCl and reduction in 400 mM NaCl was almost similar on all the treatment days (5, 10, 15 and 20d). The reduction was

higher in the plantlets subjected to 400 mM NaCl and the reduction was 38% over control plantlets. The plantlets treated with mild concentration of NaCl (100 mM) recorded 12% of enhancement in total chlorophyll content over control plantlets. The plantlets grown in 200 and 300 mM NaCl had 11 and 17% reduction in total chlorophyll content respectively compared with the control plantlets on 20d (Fig. 8).

Similar to NaCl treatment, the higher CuSO₄ concentration also had a negative impact on pigment content. The lower concentration (100 µM CuSO₄) positively influenced and it resulted in the enhancement of the total chlorophyll over control plantlets on 20d. But the higher CuSO₄ concentration (200 µM) reduced the total chlorophyll content in *V. inermis* as compared to control plantlets. The extend of reduction in total chlorophyll content was higher in plantlets subjected to NaCl compared with the plantlets subjected to CuSO₄ and both the stressors had positive influence on total chlorophyll content in its lower concentrations (Fig. 8).

4.6.2 Carotenoid content

The carotenoid content of *V. inermis* was increased in response to both NaCl and CuSO₄ treatments. The accumulation of carotenoids was observed to be progressive with increase in treatment periods. The plantlets exposed to NaCl recorded sharp and progressive increase in carotenoid content upto 300 mM NaCl. The higher accumulation in carotenoid content was observed in the leaves of plantlets grown in 300 mM NaCl and the enhancement was 59% more than control plantlets on 20d. However, the plantlets grown in 400 mM NaCl showed only minor enhancement in carotenoid content over control plantlets (Fig. 8).

The treatment with CuSO₄ also resulted in similar trend of increase in carotenoid content as that of NaCl treatment. The higher accumulation of

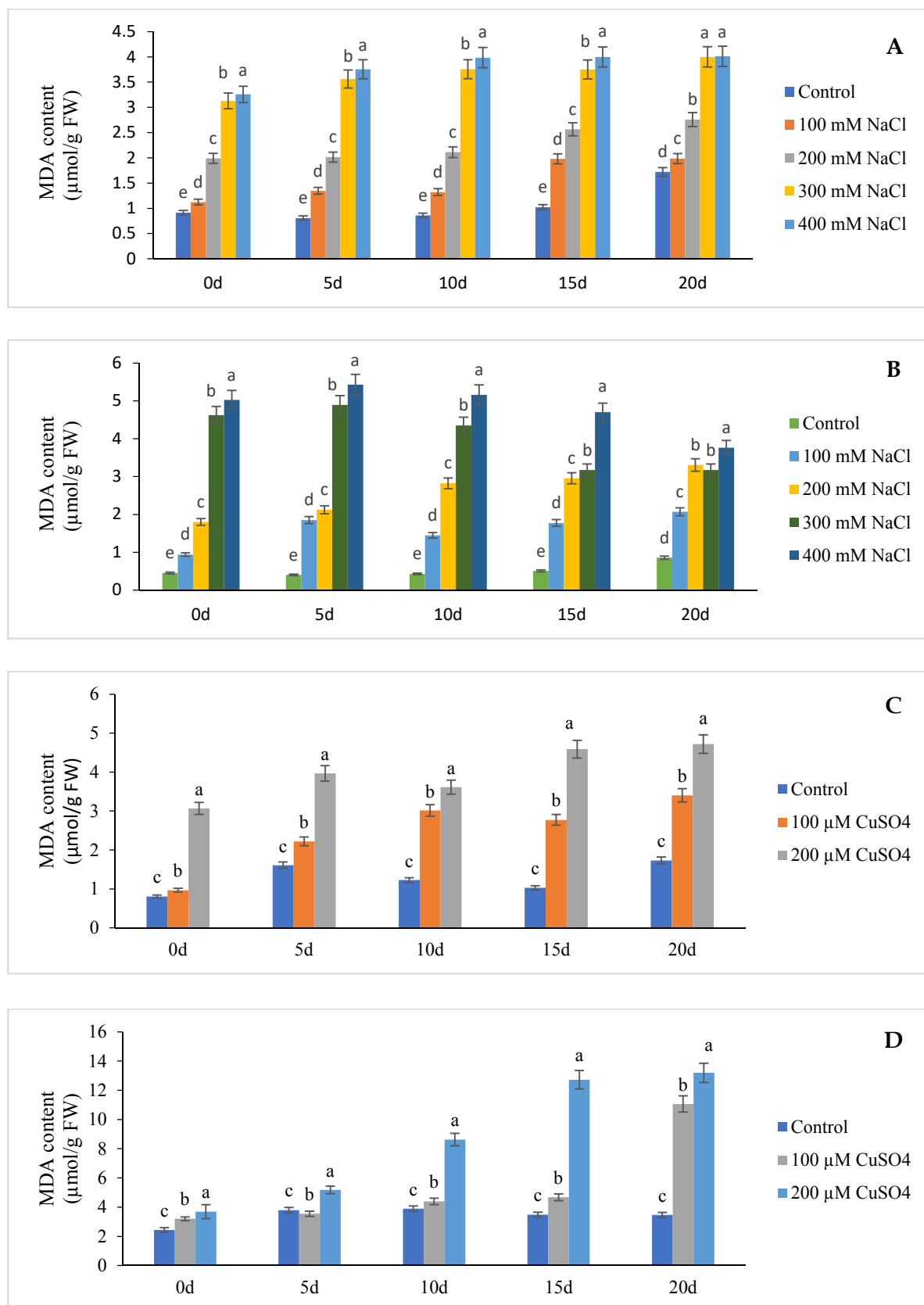


Figure 7. Malondialdehyde (MDA) in leaves (A) and roots (B) of NaCl treated as well as leaves (C) and roots (D) of CuSO₄ treated *V. inermis* plantlets. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

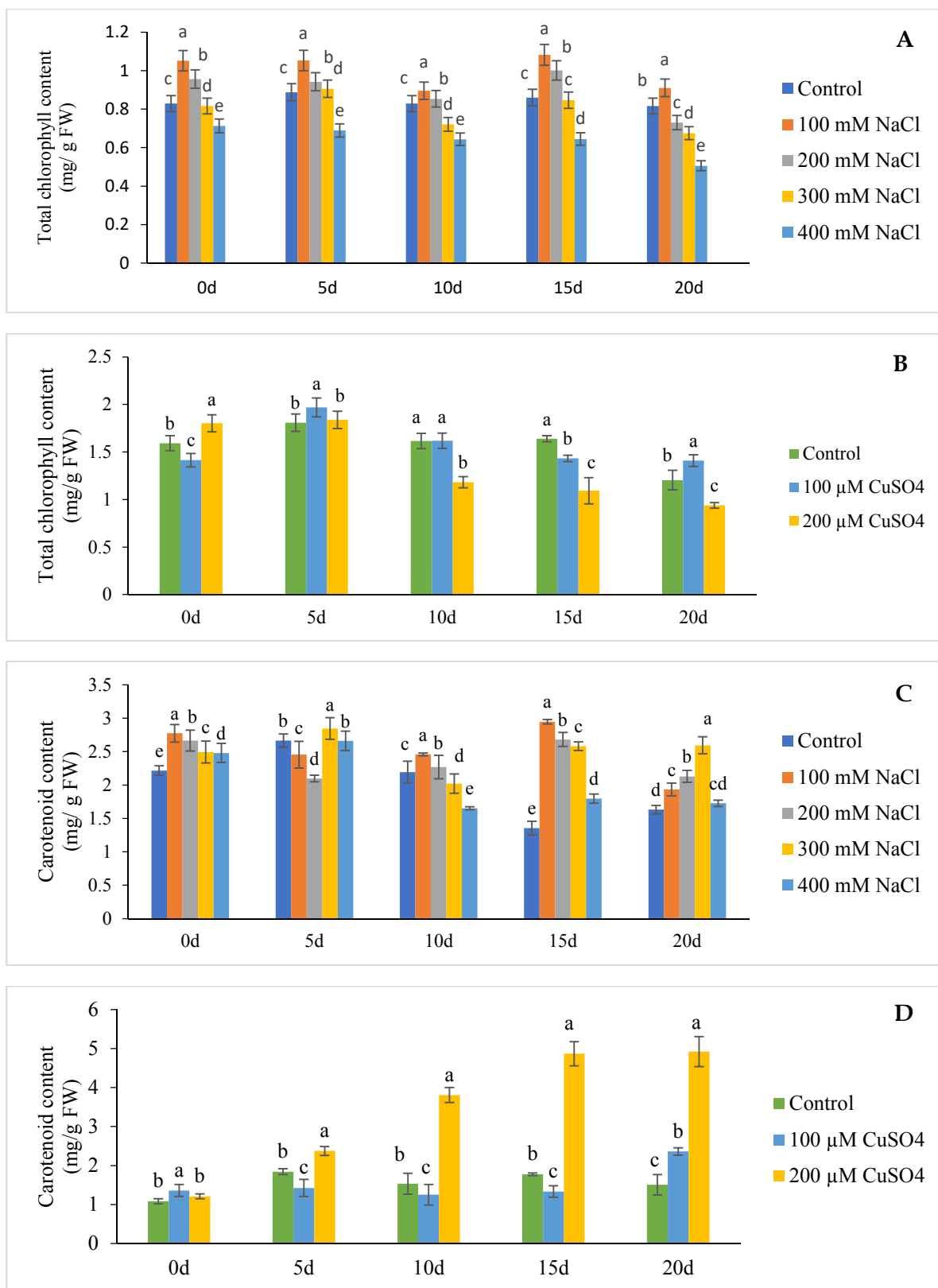


Figure 8. Total chlorophyll content (A and B) and carotenoid content (C and D) in NaCl and CuSO₄ treated leaves of *V. inermis* plantlets. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

this pigment was found in plantlets treated with 200 μM CuSO_4 and the enhancement was more prominent (227%) over control plantlets on 20d. 100 μM CuSO_4 also had a positive influence on carotenoid accumulation and it caused an increase of 56% over control plantlets on 20d. When compared to NaCl treatment, the carotenoid accumulation rate was greater in plantlets exposed to CuSO_4 (Fig. 8).

4.6.3 Chlorophyll stability index

The stability of the chlorophyll in different treatments were assessed by stability index (CSI). The CSI of the plantlets treated with different concentrations of NaCl was decreased with increasing concentration except the plantlets exposed to 100 mM NaCl. There was 11, 17 and 38% reduction in CSI was observed in the plantlets treated with 200, 300 and 400 mM NaCl as compared to the control plantlets on 20d. In contrast to this, an increment (12%) in stability of chlorophyll was found in the leaves of the plantlets subjected to 100 mM NaCl as compared to the control plantlets. In the case of plantlets treated with 100 μM CuSO_4 recorded a negligible increment in CSI as compared with control plantlets. The CSI of the plantlets grown in 200 μM CuSO_4 exhibited 41% reduction as compared to the control plantlets. As compared with the plantlets grown in NaCl, greater reduction in CSI was observed in plantlets treated with heavy metal (CuSO_4) (Fig. 9).

4.6.4 Chlorophyll *a* fluorescence induction curve and JIP parameters

The shape of the induction curves for chlorophyll fluorescence revealed the alterations in the light-dependent photosynthetic process of *V. inermis* under NaCl and CuSO_4 treatments on 20d. Chlorophyll *a* fluorescence transient OJIP curves measured in *V. inermis* plantlets treated with lower concentrations of NaCl or CuSO_4 were not significantly impacted. However, compared to the control plantlets, those subjected to

higher NaCl and CuSO₄ concentrations exhibited OJIP curve with dramatic changes. However, plantlets subjected to lower concentrations of NaCl (100 and 200 mM), and CuSO₄ (100 μ M) exhibited standard shape of the OJIP curves as that of control. Exposure of *V. inermis* to lower concentrations of NaCl (100, 200 and 300 mM) did not cause any significant change in initial fluorescence (F_o). However, in the higher concentration, F_o reduced 26% compared with the control plantlets. In the case of plantlets treated with CuSO₄, similar reduction was observed in higher concentration (200 μ M CuSO₄). In the case of maximum fluorescence (F_m), a reduction was observed and interestingly it was to the same level (29%) in plantlets treated with 400 mM NaCl and 200 μ M CuSO₄. When plantlets were subjected to 400 mM NaCl and 200 μ M CuSO₄, the F_o - F_j (F_j -chlorophyll fluorescence transient at 2 ms) phase of the transient curve decreased, however plantlets that were grown in lower concentrations of NaCl and CuSO₄ did not exhibit a pronounced decline. There was a sharp increase observed in the I step (F_I -chlorophyll fluorescence transient at 30 ms) of the plantlets exposed to 300 mM NaCl and this was not noticed in other treatments. The F_I - F_P phase was flattened in plantlets exposed to 400 mM NaCl and 200 μ M CuSO₄ but it was more prominent in plantlets grown in 400 mM NaCl compared with 200 μ M CuSO₄ (Fig. 10).

The area above the fluorescence induction curve (area) was decreased in plantlets exposed to 300 and 400 mM NaCl and the reduction was 45 and 58% respectively as compared with control plantlets. In the case of plantlets treated with lower concentrations of NaCl (100 and 200 mM), there was an increase in area and maximum increase was found in plantlets treated with 200 mM NaCl. The enhancement in the area was 50 and 60% in plantlets subjected to 100 and 200 mM NaCl respectively as compared with the control plantlets (Table 10). In the case of CuSO₄ treatment, lower concentration (100 μ M) enhances the area and the enhancement was 17% as

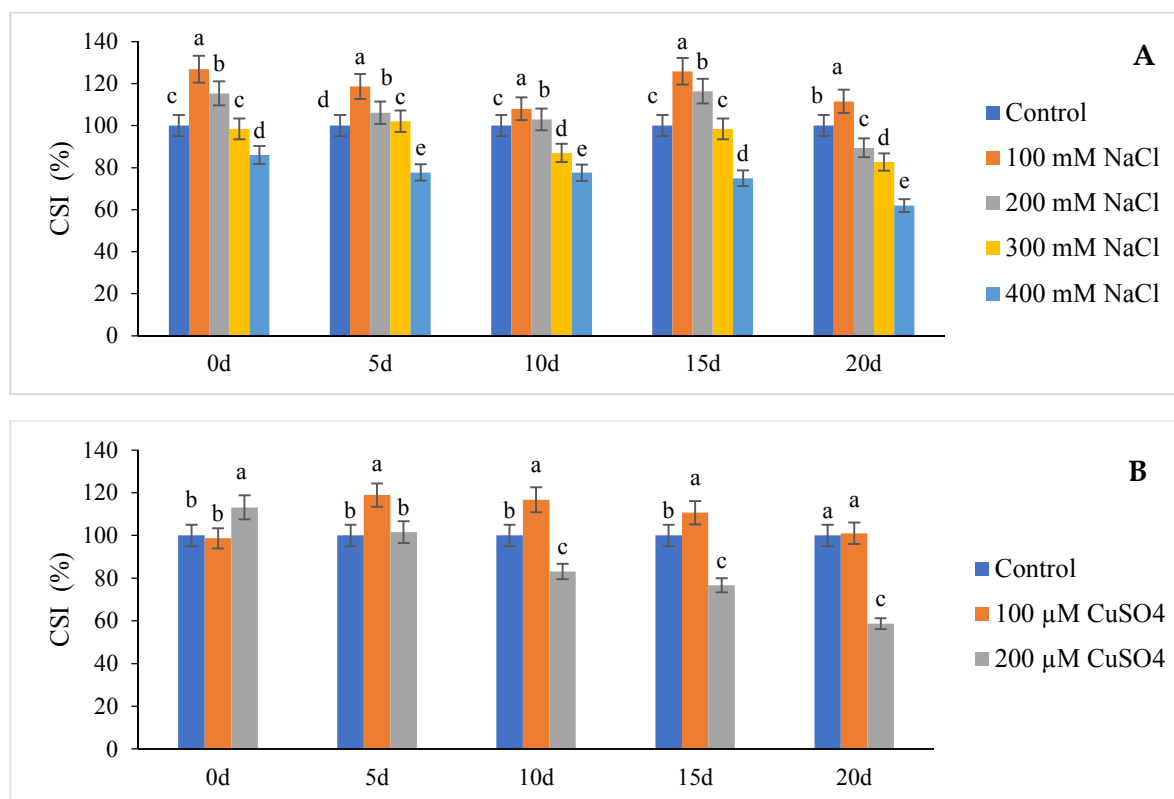


Figure 9. Chlorophyll stability index of *V. inermis* plantlets subjected to NaCl (A) and CuSO₄ (B). Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

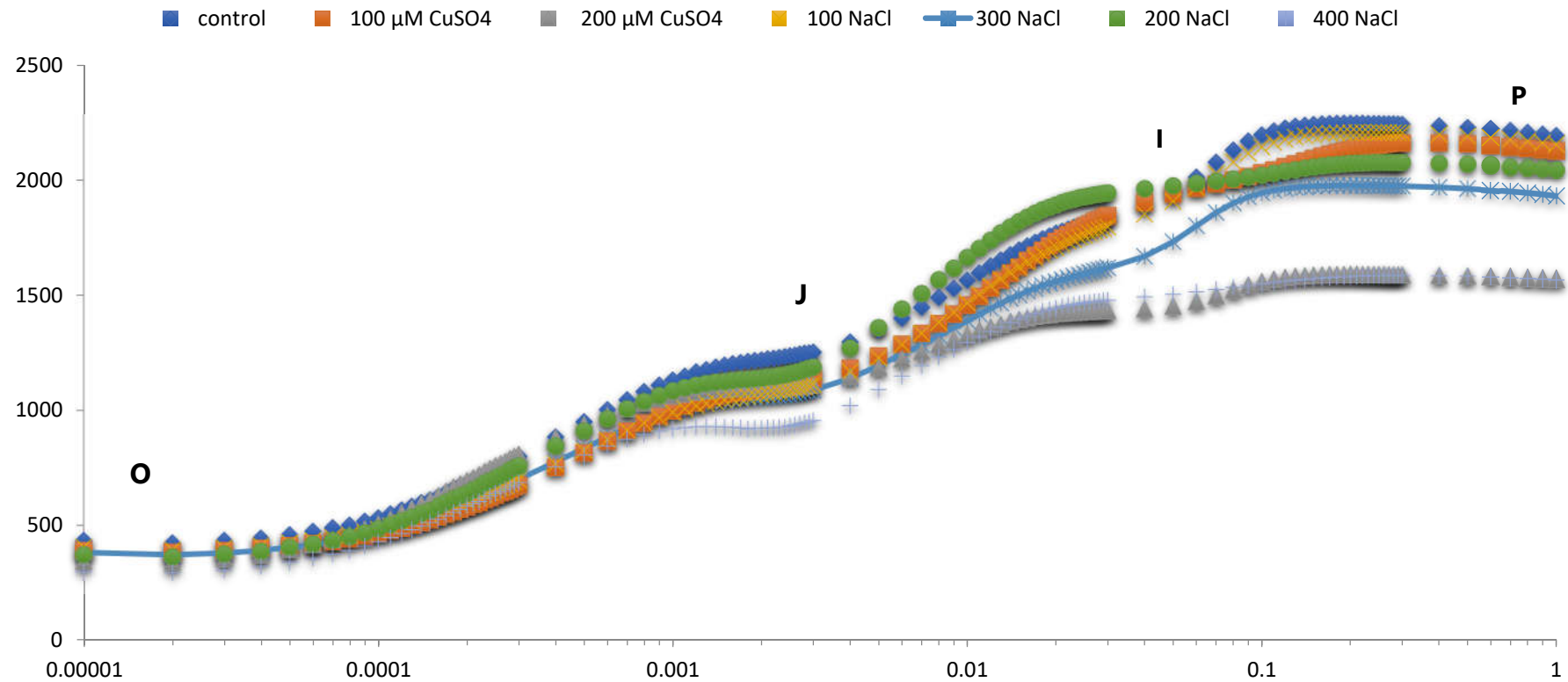


Figure 10. Chlorophyll *a* fluorescence transient curves measured in the leaves of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300, 400 mM) and CuSO₄ (0, 100 and 200 μM) on 20d.

Table 10. Modulations in different JIP parameters measured in *V. inermis* subjected to varying concentrations of NaCl. Values are expressed as mean $\pm$ SE of three different experiments. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Parameters	NaCl (mM) Treatments				
	0	100	200	300	400
Area	35015 $\pm$ 2106 ^b	37529.33 $\pm$ 2354 ^a	29120.67 $\pm$ 2596 ^c	19259 $\pm$ 1689 ^d	14815.67 $\pm$ 2139 ^e
t for Fm	196.66 $\pm$ 12 ^d	243.33 $\pm$ 17 ^b	206.66 $\pm$ 9 ^c	280 $\pm$ 10 ^a	286.66 $\pm$ 3 ^a
Fv/Fm	0.795 $\pm$ 0.006 ^b	0.836 $\pm$ 0.003 ^a	0.830 $\pm$ 0.002 ^a	0.797 $\pm$ 0.009 ^b	0.784 $\pm$ 0.010 ^c
Fv	1788.66 $\pm$ 17 ^b	1847.33 $\pm$ 58 ^a	1642.33 $\pm$ 87 ^c	1645.33 $\pm$ 28 ^c	1247 $\pm$ 126 ^d
Fo	458.66 $\pm$ 13 ^a	361 $\pm$ 17 ^c	335.33 $\pm$ 13 ^e	417.66 $\pm$ 19 ^b	338.66 $\pm$ 21 ^d
Fm	2247.33 $\pm$ 8 ^a	2208.33 $\pm$ 74 ^b	1977.66 $\pm$ 100 ^d	2063 $\pm$ 15 ^c	1585.66 $\pm$ 144 ^e
Fv/Fo	3.90 $\pm$ 0.15 ^c	5.12 $\pm$ 0.11 ^a	4.89 $\pm$ 0.07 ^b	3.945 $\pm$ 0.26 ^c	3.67 $\pm$ 0.22 ^d
SFI	2.04 $\pm$ 0.14 ^b	2.504 $\pm$ 20 ^a	2.03 $\pm$ 0.08 ^b	1.974 $\pm$ 0.18 ^c	1.39 $\pm$ 0.18 ^d
Sm	19.55 $\pm$ 0.99 ^b	20.37 $\pm$ 1.28 ^a	17.71 $\pm$ 0.85 ^c	12.68 $\pm$ 0.88 ^d	11.77 $\pm$ 0.54 ^e
N	34.90 $\pm$ 1.24 ^a	31.92 $\pm$ 1.38 ^b	29.90 $\pm$ 1.02 ^c	28.08 $\pm$ 1.59 ^c	22.50 $\pm$ 0.53 ^d
DF abs	0.371 $\pm$ 0.06 ^c	0.646 $\pm$ 0.06 ^a	0.487 $\pm$ 0.03 ^b	0.301 $\pm$ 0.07 ^d	0.134 $\pm$ 0.09 ^e
DF Total	0.185 $\pm$ 0.04 ^c	0.398 $\pm$ 0.06 ^a	0.282 $\pm$ 0.07 ^b	-0.523 $\pm$ 0.12 ^e	-0.574 $\pm$ 0.08 ^d
PI abs	2.40 $\pm$ 0.036 ^c	4.53 $\pm$ 0.62 ^a	3.091 $\pm$ 0.22 ^b	2.06 $\pm$ 0.39 ^d	1.42 $\pm$ 0.26 ^e
PI total	1.55 $\pm$ 0.15 ^c	2.55 $\pm$ 0.35 ^a	1.97 $\pm$ 0.32 ^b	0.328 $\pm$ 0.10 ^d	0.276 $\pm$ 0.05 ^e
Phi(Eo)	0.457 $\pm$ 0.02 ^c	0.517 $\pm$ 0.021 ^a	0.465 $\pm$ 0.011 ^b	0.439 $\pm$ 0.017 ^d	0.417 $\pm$ 0.012 ^e
Psi(Eo)	0.574 $\pm$ 0.021 ^b	0.618 $\pm$ 0.021 ^a	0.561 $\pm$ 0.011 ^c	0.551 $\pm$ 0.014 ^d	0.531 $\pm$ 008 ^e
Phi(Po)	0.795 $\pm$ 0.006 ^b	0.836 $\pm$ 0.003 ^a	0.830 $\pm$ 0.002 ^a	0.797 $\pm$ 0.009 ^b	0.784 $\pm$ 0.11 ^c

compared with control plantlets. However, the plantlets grown in 200 μM CuSO_4 exhibited 56% reduction in area as compared to control plantlets (Table 11). The reduction of area in higher concentration of NaCl and CuSO_4 treated plantlets exhibit almost a similar trend. Whereas the lower concentrations of both treatments influence positively and resulted in the increase of area above the fluorescence induction curve.

Tf(max) represents the time taken to reach Fm. In NaCl treated plantlets, the rate of increment was highest in 300 and 400 mM and was higher by 42 and 46% respectively as compared with the control plantlets. The increment in Tf(max) was also observed in other NaCl treatments, but it was negligible in plantlets exposed to 100 and 200 mM NaCl as compared with the control plantlets (Table 10). The exposure of *V. inermis* to CuSO_4 resulted in the enhancement of Tf(max) and 84% increase was recorded in plantlets grown in the 200 μM . However, the increment in Tf(max) of plantlets grown in 100 μM CuSO_4 was very negligible (Table 11). Both NaCl and CuSO_4 treatments caused an increase in Tf(max). However, compared to control plantlets, exposure to heavy metal (Cu) had a more profound impact than NaCl.

The maximum quantum yield of PSII (Fv/Fm) of the control plantlets was 0.795 ± 0.039 . Similar value of Fv/Fm was maintained in plantlets treated with 300 mM NaCl. However, slight increment was recorded in plantlets subjected to lower NaCl concentrations (100 and 200 mM NaCl). The value of Fv/Fm in plantlets exposed to higher NaCl concentration (400 mM) was slightly lesser and it was 0.784 ± 0.039 (Table 10). The exposure to mild concentration of CuSO_4 did not cause any reduction and Fv/Fm was maintained as like that of control. The plantlets treated with 200 μM CuSO_4 caused a reduction in Fv/Fm to 0.750 ± 0.037 . In the case of Fv/Fm, the higher reduction was due to CuSO_4 treatment than NaCl (Table 10 and 11).

The maximal variable Chl *a* fluorescence ($F_v = F_m - F_o$), reduced drastically in response to 400 mM NaCl treatment in *V. inermis* and the reduction was 30% as compared to the control plantlets. At the same time, the plantlets grown in 100 and 200 mM NaCl exhibited an increase in F_v and it was 15 and 18% respectively as compared to the control plantlets (Table 10). On exposure to 200 μ M CuSO_4 , the F_v reduced by 33% as compared with the control plantlets and the reduction was very negligible in lower CuSO_4 concentration (100 μ M) (Table 11).

The parameter F_v/F_o represents the maximum efficiency of water splitting complex. The plantlets exposed to 400 mM NaCl exhibited a reduction in F_v/F_o . At the same time, the plantlets grown in 100 and 200 mM NaCl showed an increment in efficiency of water splitting complex (31 and 25% respectively) compared to control plantlets (Table 10). The plantlets grown in lower CuSO_4 concentration had negligible enhancement in F_v/F_o . However, the efficiency of the water splitting complex was reduced (23%) in plantlets grown in 200 μ M CuSO_4 as compared to the control plantlets (Table 11). In comparison to NaCl treatments, plantlets exposed to 200 μ M CuSO_4 showed a notable decline in F_v/F_o values.

The structure function index or plant vitality index of PSII (SFI) of plantlets exposed to NaCl was decreased in plantlets exposed to 400 mM NaCl. But in the case of plantlets subjected to 300 mM NaCl, the value were maintained same as that of control. The treatment of lowest NaCl concentration (100 mM), caused an increase in SFI (23%) than control plantlets (Table 10). On exposure to 200 μ M CuSO_4 , the SFI reduced by 51% as compared with the control plantlets. At the same time, exposure of *V. inermis* to 100 μ M CuSO_4 resulted in the enhancement of SFI and it was 27% over control plantlets (Table 11). The intensity of reduction in SFI was greater due to heavy metal (CuSO_4) treatment than the NaCl.

Table 11. Modulations in different JIP parameters measured in *V. inermis* subjected to varying concentrations of CuSO₄. Values are expressed as mean $\pm$ SE of three different experiments. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Parameters	CuSO ₄ (μ M) Treatments		
	0	100	200
Area	35015 $\pm$ 2106 ^b	40796.67 $\pm$ 1398 ^a	15509 $\pm$ 1641 ^c
t for Fm	196.66 $\pm$ 12 ^c	363.33 $\pm$ 68 ^a	206.66 $\pm$ 12 ^b
Fv/Fm	0.795 $\pm$ 0.006 ^b	0.809 $\pm$ 0.004 ^a	0.750 $\pm$ 0.010 ^c
Fv	1788.66 $\pm$ 17 ^a	1751 $\pm$ 92 ^a	1195.33 $\pm$ 86 ^b
Fo	458.66 $\pm$ 13 ^a	411 $\pm$ 11 ^b	394.33 $\pm$ 8 ^c
Fm	2247.33 $\pm$ 8 ^a	2162 $\pm$ 101 ^b	1589.66 $\pm$ 94 ^c
Fv/Fo	3.90 $\pm$ 0.15 ^b	4.25 $\pm$ 0.13 ^a	3.02 $\pm$ 0.15 ^c
SFI	2.04 $\pm$ 0.14 ^b	2.59 $\pm$ 0.04 ^a	0.992 $\pm$ 0.12 ^c
Sm	19.55 $\pm$ 0.99 ^b	23.47 $\pm$ 0.53 ^a	12.91 $\pm$ 1.79 ^c
N	34.90 $\pm$ 1.24 ^b	36.07 $\pm$ 2.45 ^a	30.48 $\pm$ 0.77 ^c
DF abs	0.371 $\pm$ 0.06 ^b	0.541 $\pm$ 0.02 ^a	-0.176 $\pm$ 0.09 ^c
DF Total	0.185 $\pm$ 0.04 ^a	0.170 $\pm$ 0.06 ^a	-0.496 $\pm$ 0.03 ^b
PI abs	2.40 $\pm$ 0.036 ^b	3.48 $\pm$ 0.16 ^a	0.694 $\pm$ 0.12 ^c
PI total	1.55 $\pm$ 0.15 ^a	1.50 $\pm$ 0.19 ^a	0.321 $\pm$ 0.02 ^b
Phi(Eo)	0.457 $\pm$ 0.02 ^b	0.492 $\pm$ 0.006 ^a	0.308 $\pm$ 0.021 ^c
Psi(Eo)	0.574 $\pm$ 0.021 ^b	0.608 $\pm$ 0.005 ^a	0.410 $\pm$ 0.023 ^c
Phi(Po)	0.795 $\pm$ 0.006 ^b	0.809 $\pm$ 0.004 ^a	0.750 $\pm$ 0.011 ^c

The parameter S_M represents the multiple turnover of Q_A reductions. It was drastically reduced in the plantlets subjected to 300 and 400 mM NaCl and the reduction was 35 and 40% respectively as compared with the control plantlets. Exposure to the other two NaCl concentrations (100 and 200 mM) resulted in a slight increase of S_M in plantlets treated with 100 mM NaCl (Table 10). Exposure of plantlets to mild CuSO_4 (100 μM) caused an increment in S_M and it was 20% higher than control plantlets. In contrast to this, exposure to 200 μM CuSO_4 resulted in the reduction of S_M and it was 34% lower than control plantlets. A pronounced decrease in multiple turnover of Q_A reduction was observed in plantlets exposed to NaCl than the plantlets exposed to CuSO_4 (Table 11).

The N represents the turnover number of Q_A , more specifically the number of times Q_A was reduced from 0 to $T_f(\text{max})$. The N was reduced progressively in plantlets of *V. inermis* in response to NaCl treatments and the reduction was maximum in plantlets grown in 400 mM NaCl. About 36% reduction of N was observed in higher NaCl concentration (400 mM) compared with the control plantlets (Table 10). The 200 μM CuSO_4 induced reduction was only 13% in plantlets over control plantlets and the lower CuSO_4 treatment (100 μM) did not cause any reduction in N and it was similar to that in control plantlets (Table 11).

The PSII relative driving force index on absorption basis is represented as DF_{ABS} . The DF_{ABS} was reduced in response to NaCl treatments of higher concentrations (300 and 400 mM) and it increased in plantlets treated with lower concentrations (100 and 200 mM). The reduction in DF_{ABS} was 19 and 64% in *V. inermis* in response to 300 and 400 mM NaCl respectively as compared with the control plantlets. The higher increment in DF_{ABS} was found in plantlets treated with 100 mM NaCl and was 74% higher than control plantlets (Table 10). The 100 μM CuSO_4 treatment positively

influenced DF_{ABS} and resulted in an increase of 46% than control plantlets. However, 200 μ M modulates the DF_{ABS} negatively and the reduction was 147% as compared with the control plantlets. The higher reduction in DF_{ABS} was found in plantlets treated with higher $CuSO_4$ (200 μ M) and it was higher than any of the NaCl treatments. Similar to DF_{ABS} , DF_{total} of the plantlets treated with higher NaCl (400 mM) and $CuSO_4$ (200 μ M) also reduced and reduction was in the range 409 and 367% as compared with the control plantlets. The reduction in DF_{total} was found to be higher than DF_{ABS} (Table 11).

The parameter PI_{ABS} indicates performance index on absorption basis and it decreased in response to 300 and 400 mM NaCl and the reduction was found to be 14 and 41% respectively as compared to control plantlets. The increment in PI_{ABS} than control plantlets was found in plantlets treated with 100 and 200 mM NaCl and it was 88 and 28% respectively over control plantlets (Table 10). Similar kind of increment in PI_{ABS} was found in plantlets treated with 100 μ M $CuSO_4$ and was 45% more than control plantlets. At the same time, plantlets grown in 200 μ M $CuSO_4$ exhibited 71% reduction as compared to control plantlets. The reduction level was comparatively higher in $CuSO_4$ treatment as compared to NaCl treatment. Total PI (PI_{total}), measuring the performance index of electron flux to the final PSI electron acceptors showed reductions in response to higher NaCl treatments (300 and 400 mM) and the reduction was 82 and 79% respectively than control plantlets. The plantlets grown in lower NaCl concentrations recorded an enhancement in the PI_{total} and it was upto 65% (in 100 mM NaCl) and 27% (in 200 mM NaCl) than control plantlets. The PI_{total} of the plantlets treated with 200 μ M $CuSO_4$ exhibited 79% reduction than control plantlets and in plantlets exposed to 100 μ M there was a major change as compared to control plantlets (Table 11). As far as the PI_{total} is concerned, the responses

were almost similar in plantlets treated with higher NaCl and CuSO₄ concentrations.

The parameters such as PHI(Eo) [ϕ Eo], PSI(Eo) [Ψ Eo], and PHI(Po) [ϕ Po] represents quantum yield (at $t = 0$) for electron transport from Q_A^- to plastoquinone, the probability (at $t=0$) that a trapped exciton moves an electron into the electron transport chain beyond Q_A^- and the maximum quantum yield of primary PSII photochemistry at $t = 0$ respectively. PHI(Eo) was reduced (90%) in *V. inermis* on exposure to 100 mM NaCl as compared to control plantlets. However, there was no major change in values for this parameters in plantlets treated with 100 and 200 mM NaCl as compared with control. The plantlets exposed to 200 μ M CuSO₄ exhibited higher reduction in PHI(Eo) and the value was 33% less than the control plantlets. An 8% increment in PSI(Eo) was observed in plantlets treated with 100 mM NaCl and a reduction of 8% was observed in plantlets treated with 400 mM NaCl over control plantlets (Table 10). The reductions in plantlets treated with 200, 300 mM NaCl was negligible (2-4%) over control plantlets. Exposure to 200 μ M CuSO₄ seems to negatively affect PSI(Eo) and it was reduced by 29% in comparison with control plantlets. PHI(Po) showed lesser yield reduction in maximum quantum yield even under higher concentrations of NaCl and CuSO₄ treated plantlets over control plantlets (Table 11). The mild NaCl and CuSO₄ treated plantlets exhibits a minor increase (2 -5%) of PHI(Po) than control plantlets.

The phenomenological energy flux parameter ABS/CS_M, representing absorption of energy per excited cross section (CS) approximated by Fm was reduced in response to NaCl treatment. It showed a gradual decrease with increasing concentration of NaCl and exhibited maximum reduction in plantlets treated with 400 mM NaCl (26%). The plantlets treated with 200 μ M CuSO₄ also showed reduction (24%) as compared to control plantlets

(Fig. 12 and Table 12). However, only a minor increase was found in plantlets treated with 100 μM CuSO_4 as compared with control plantlets (Fig. 12 and Table 12). The excitation energy flux trapped by PSII per CS approximated by F_m was represented as TRo/CS_M . The reduction of the parameter TRo/CS_M in plantlets treated with 200, 300 and 400 mM NaCl was to the extent of 10-38% as compared with control plantlets. However, the plantlets exposed to 100 mM NaCl exhibited almost similar level of TRo/CS_M as that of control (Fig. 11 and Table 12). Similar kind of observation was found in plantlets treated with CuSO_4 . Higher reduction was observed in plantlets exposed to 200 μM CuSO_4 as compared to the control plantlets. The plantlets grown in 100 μM CuSO_4 exhibited almost similar level of TRo/CS_M as that of control (Fig. 12 and Table 12).

The electron transport per CS approximated by F_m was represented as ETo/CS_M . ETo/CS_M was negatively impacted by the NaCl treatments and the decrease was 51 and 53% in *V. inermis* exposed to 300 and 400 mM NaCl, respectively. The ETo/CS_M of the plantlets treated with 100 mM NaCl increased and was sustained at a level that was nearly identical to that of the control plantlets (Fig. 11 and Table 12). The *V. inermis* treated with 200 μM CuSO_4 exhibited 50% reduction in ETo/CS_M as compared with the control plantlets (Fig. 11 and Table 12). The dissipated energy is flux per CS approximated by F_m is represented as DIO/CS_M . The plantlets grown in 400 mM NaCl exhibited an increase in DIO/CS_M as compared to the control plantlets. Similarly, plantlets grown in 100 and 200 μM CuSO_4 exhibited 16 and 29% increase in DIO/CS_M as compared with control plantlets (Fig. 12 and Table 12). The reduction of end acceptors at PSI electron acceptor side per CS approximated by F_m is represented as REo/CS_M . In the case of NaCl treatment, higher reduction in REo/CS_M was observed in plantlets exposed to 300 and 400 mM NaCl and the reduction was 38 and 50% respectively over control plantlets. An increase of REo/CS_M was observed in plantlets

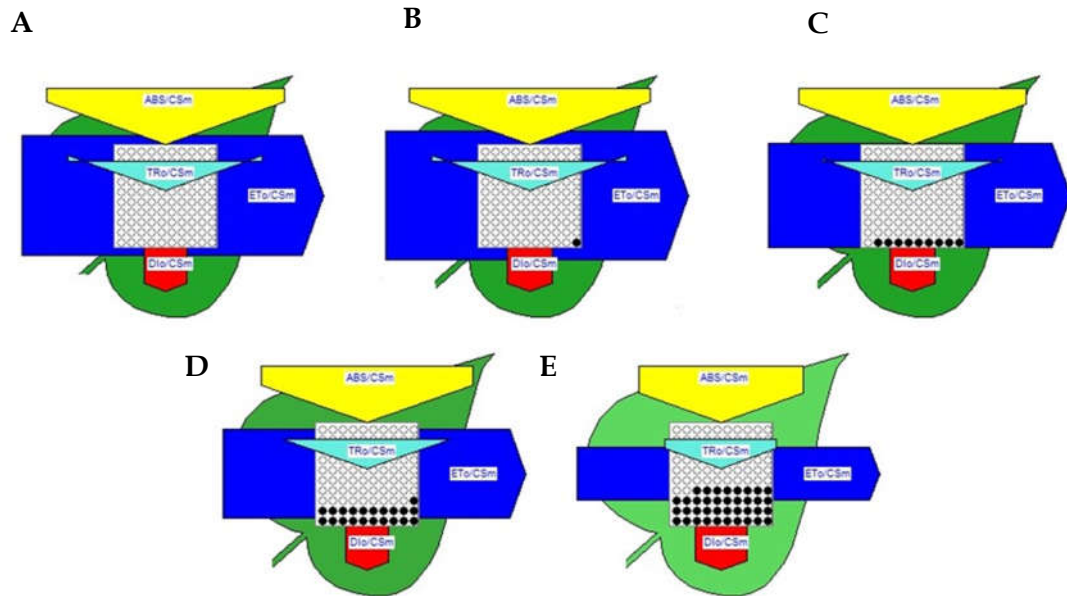


Figure 11. Leaf pipeline model showing the proportion of phenomenological energy flux parameters (calculated per cross section approximated by maximal fluorescence) in *V. inermis* plantlets subjected to varying concentrations of NaCl (A-0, B-100, C-200, D-300 and E-400 mM) on 20d. The value of each parameter can be seen as relative change in the width of each arrow.

Table 12. Phenomenological energy flux parameters (calculated per cross section approximated by maximal fluorescence) in *V. inermis* plantlets subjected to varying concentrations of NaCl and CuSO₄. Values are expressed as mean $\pm$ SE of three different experiments. Different alphabetical letters indicate significant difference between means (Duncan's test $p \leq 0.05$).

Parameters	Treatments							
	NaCl (mM)					CuSO ₄ (μ M)		
	0	100	200	300	400	0	100	200
ABS/CSm	2142 $\pm$ 107 ^a	2064 $\pm$ 103 ^b	1872 $\pm$ 94 ^c	1785 $\pm$ 89 ^d	1580 $\pm$ 79 ^e	2142 $\pm$ 107 ^a	2192 $\pm$ 110 ^a	1608 $\pm$ 80 ^b
TRo/CSm	1750 $\pm$ 88 ^a	1733 $\pm$ 87 ^a	1575 $\pm$ 79 ^b	1481 $\pm$ 74 ^c	1160 $\pm$ 58 ^d	1750 $\pm$ 88 ^a	1739 $\pm$ 87 ^a	1103 $\pm$ 55 ^b
ETo/CSm	1077 $\pm$ 54 ^a	1105 $\pm$ 55 ^a	885 $\pm$ 44 ^b	530 $\pm$ 27 ^c	502 $\pm$ 25 ^d	1077 $\pm$ 54 ^b	1109 $\pm$ 55 ^a	535 $\pm$ 27 ^c
DIo/CSm	392 $\pm$ 20 ^c	304 $\pm$ 17 ^d	331 $\pm$ 20 ^b	397 $\pm$ 15 ^e	420 $\pm$ 21 ^a	392 $\pm$ 20 ^c	453 $\pm$ 23 ^b	505 $\pm$ 26 ^a

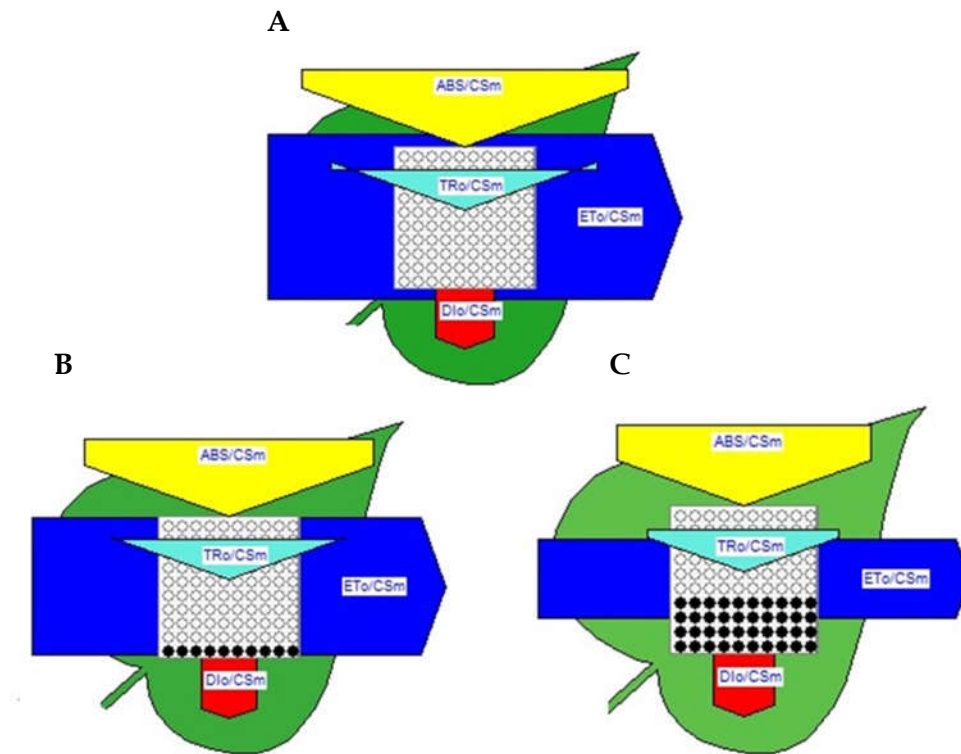


Figure 12. Leaf pipeline model showing the proportion of phenomenological energy flux parameters (calculated per cross section approximated by maximal fluorescence) in *V. inermis* plantlets subjected to varying concentrations of $CuSO_4$ (A-0, B-100 and C-200 μM) on 20d. The value of each parameter can be seen as relative change in the width of each arrow.

treated with 100 mM NaCl and was 17% higher than control plantlets. The treatment of 100 and 200 μ M CuSO₄ in *V. inermis* resulted in 22 and 80% reduction as compared to the control plantlets (Fig. 12 and Table 12).

4.6.5 Leaf gas exchange parameters

4.6.5.1 Net photosynthetic rate (A_{net})

Treatment of plantlets with higher concentrations of NaCl and CuSO₄ retarded the net photosynthetic rate (A_{net}) of *V. inermis*. The mild concentrations of NaCl and CuSO₄ did not change A_{net} severely. The treatment with 300 and 400 mM NaCl reduces the A_{net} upto 16 and 45 % as compared to control plantlets. Where as in plantlets treated with 200 μ M CuSO₄, there was 40% reduction in A_{net} as compared with control plantlets (Fig.13).

4.6.5.2 Stomatal conductance (g_s)

A progressive decrease in g_s was found in NaCl and CuSO₄ treatments. The plantlets treated with 200 μ M CuSO₄ exerted a profound reduction on stomatal conductance (g_s) in *V. inermis* than NaCl and it was 46% lower than control plantlets. The treatment of higher NaCl concentration (400 mM) caused a reduction (40%) in g_s as compared to control plantlets. A similar trend of decrease (22-23%) was also found in plantlets subjected to 200 and 300 mM NaCl as compared with control plantlets. The lower NaCl and CuSO₄ treatments cause negligible reduction (5%) in stomatal conductance than control plantlets (Fig.13).

4.6.5.3 Transpiration (Tr)

The Tr of the plantlets was reduced in response to different treatments of NaCl and CuSO₄. Higher reduction in Tr was observed in the plantlets treated with 300 and 400 mM NaCl (69%) over control plantlets. Similar rate of reduction in Tr was also observed in plantlets treated with 200 μ M CuSO₄ (62%) as compared with the control plantlets (Fig.14).

4.6.5.4 Water use efficiency (WUE)

The plantlets exposed to NaCl and CuSO₄ exhibited an increase in WUE except the plantlets grown in 100 μ M CuSO₄. Prominent increase in WUE was found in plantlets grown in 300 mM NaCl and was 170% over control plantlets. The other NaCl treatments (100, 200 and 400 mM) exerts an increase of 63 to 86% over control plantlets. The lower concentration of CuSO₄ exhibits a reduction (20%) in WUE over control plantlets. At the same time, WUE of *V. inermis* exposed to 200 μ M CuSO₄ was increased (66%) over control plantlets (Fig.14).

4.6.6 Photosystem activities

4.6.6.1 Photosystem (PS) I activity

The PSI activity was reduced in response to the exposure of NaCl except the plantlets treated with 100 mM NaCl. The reduction was higher in plantlets treated with 400 mM NaCl and was 86% as compared with control plantlets on 20d. The reduction in PSI activities was 46 and 76% in plantlets treated with 200 and 300 mM NaCl respectively as compared with control plantlets. Similar observation was also found in plantlets treated with CuSO₄. Exposure to 200 μ M CuSO₄ caused 77% reduction in PSI activity as compared with control plantlets (Fig. 15).

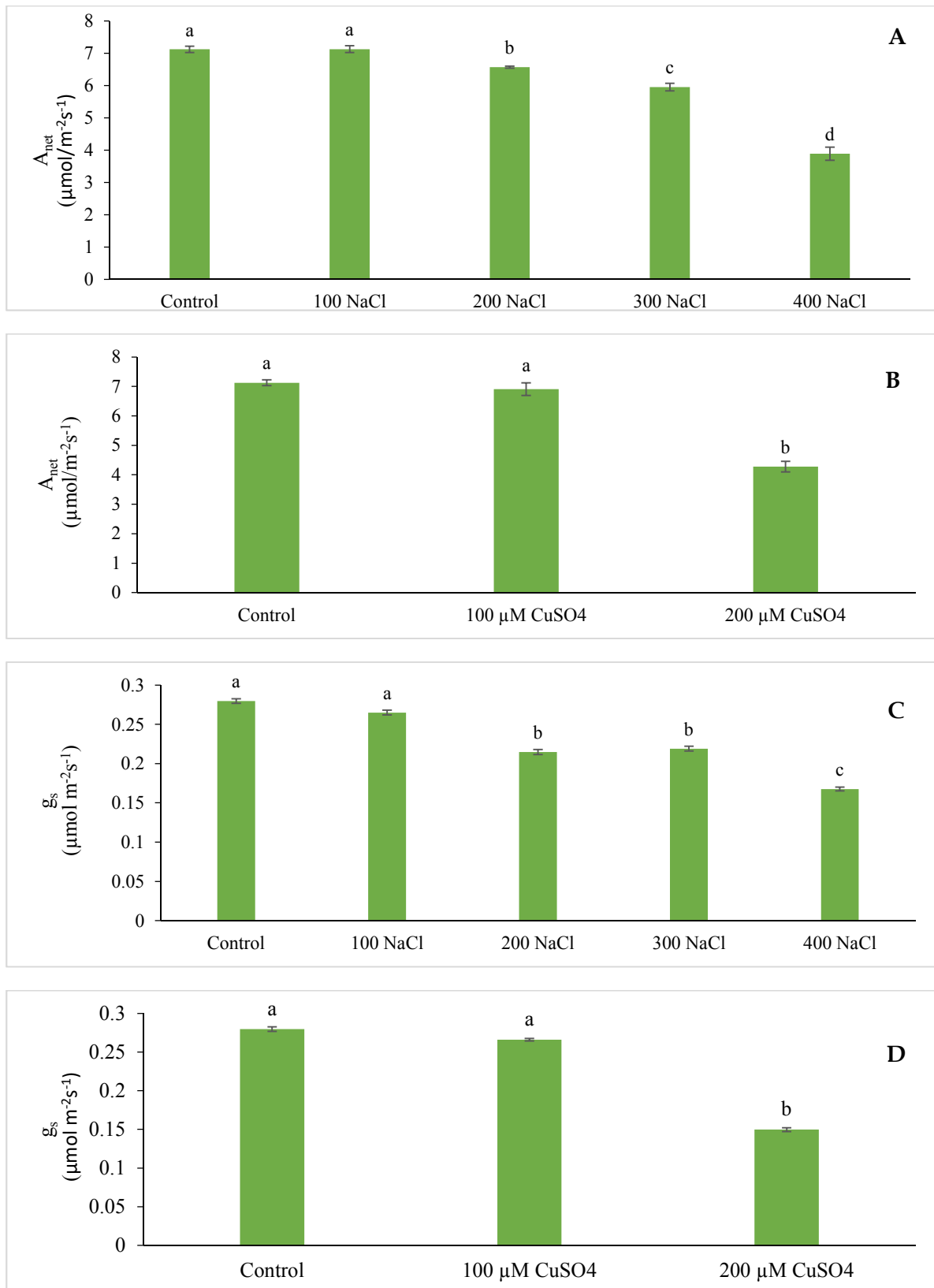


Figure 13. Net photosynthetic rate (A_{net}) (A and B) and stomatal conductance (g_s) (C and D) in NaCl and CuSO_4 treated leaves of *V. inermis* plantlets on 20d. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

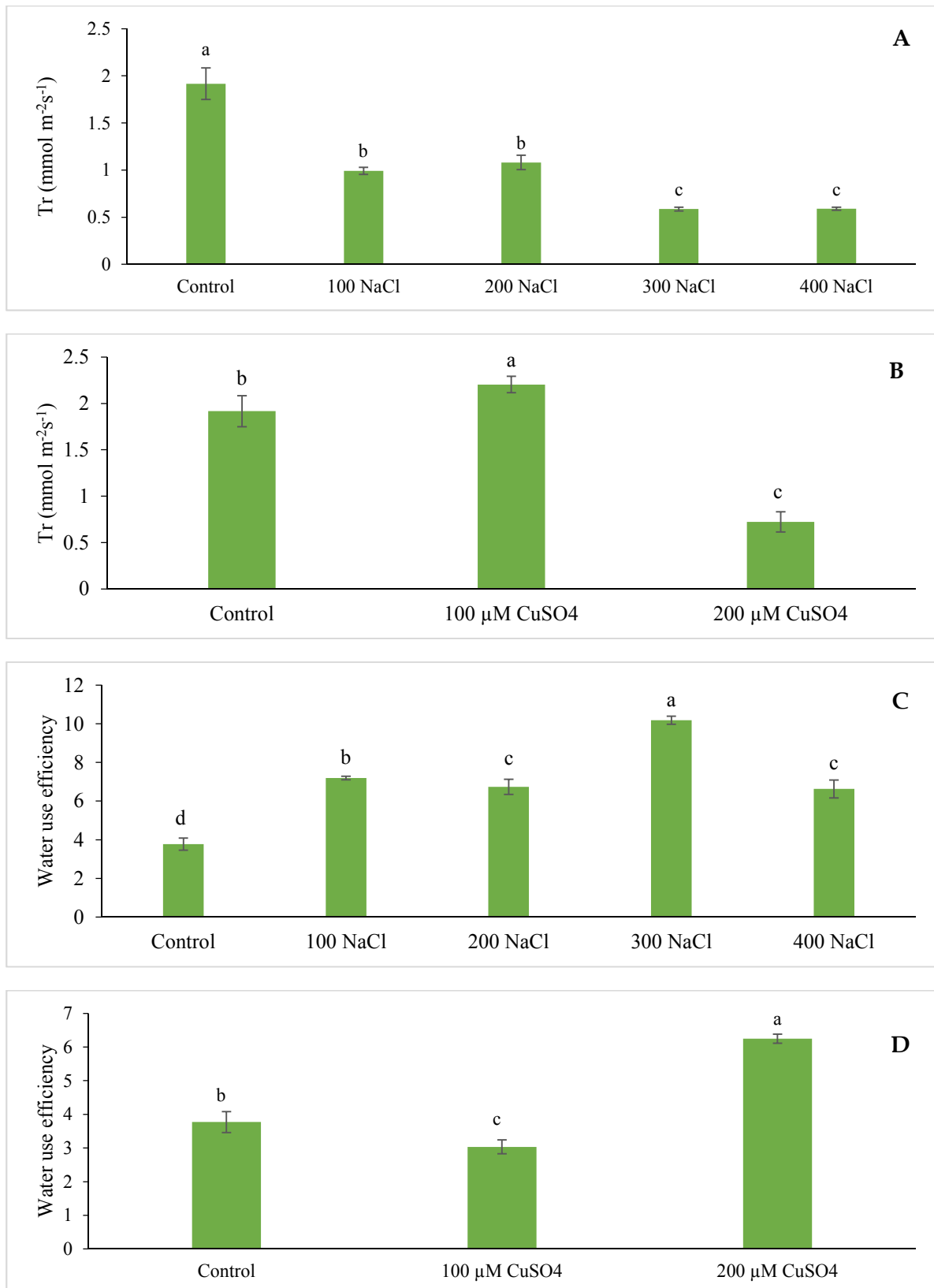


Figure 14. Transpiration rate (Tr) (A and B) and water use efficiency (WUE) (C and D) in NaCl and CuSO₄ treated leaves of *V. inermis* plantlets on 20d. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

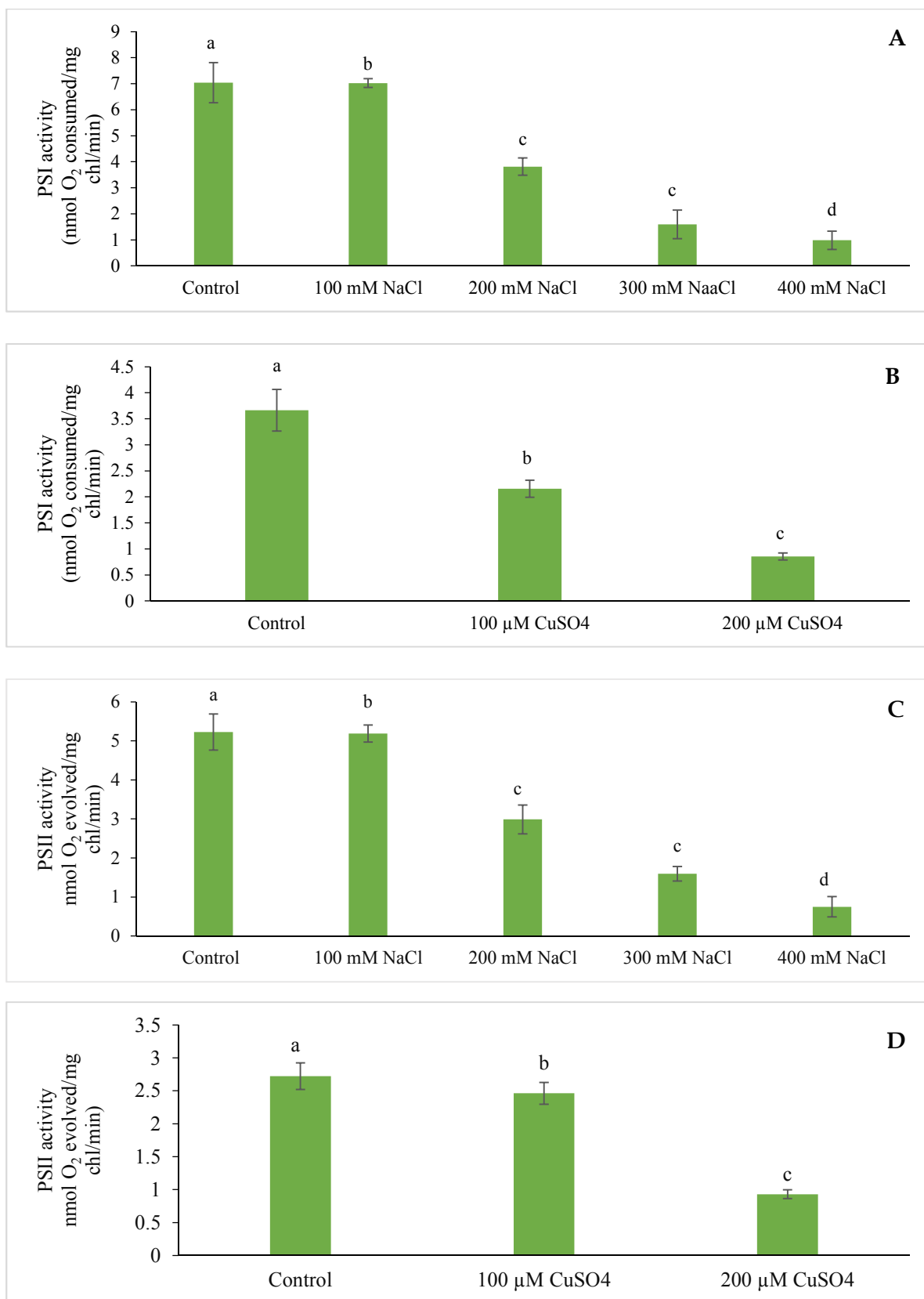


Figure 15. PSI (A and B) and PSII activities (C and D) in leaves of *V. inermis* plantlets treated with NaCl and CuSO₄ on 20d. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

4.6.6.2 Photosystem (PS) II activity

The plantlets treated with higher concentration of NaCl reduced the PSII activity upto 86% as compared with control plantlets. The lower concentration of NaCl had no effect on PSII activity. The reduction in PSII activities was 43 and 70% in plantlets treated with 200 and 300 mM NaCl treated plantlets respectively as compared with control plantlets. The PSII activity was also reduced in 200 μ M CuSO₄ treatment. The lower CuSO₄ (100 μ M) had less effect on PSII activity and it was less than 10%. Exposure to 200 μ M CuSO₄ caused 66% reduction in PSII activity as compared with control plantlets (Fig. 15).

4.7 Osmolality and osmolytes

4.7.1 Osmolality

The leaves and roots of plantlets exhibited a substantial rise in osmolality on exposure to NaCl in comparison to control plantlets. The leaves of the plantlets treated with 400 mM NaCl showed the greatest increase, which was 203% as compared with the control plantlets. The plantlets grown in 100, 200, and 300 mM NaCl exhibited an increase of 32, 59 and 99% respectively in osmolality as compared with the control plantlets. In the case of roots, lower NaCl concentration (100 mM) did not cause any increase in osmolality and it maintained as like that of control plantlets. But the plantlets treated with 200, 300 and 400 mM NaCl augment the osmolality level by 26, 33 and 57% respectively as compared with the control plantlets (Fig. 16).

The CuSO₄ treatment in *V. inermis* resulted in gradual increase of osmolality and it positively progresses with increase in the period of treatment in roots and leaves. The plantlets treated with 100 and 200 μ M CuSO₄ resulted in an increase of osmolality to 43 and 96% (in leaves) and 20

and 99% (in roots) as compared with the control plantlets. The enhancement in osmolality was greater in the leaves of NaCl treated plantlets compared with leaves of plantlets treated with CuSO₄ (Fig. 16).

4.7.2 Total soluble sugars

The total soluble sugars content in the leaves and roots of NaCl treated plantlets was varying considerably. The accumulation of sugars was increased in leaves of NaCl treated plantlets except in the case of plantlets grown in 100 mM NaCl. The significant increase was observed in the leaves of the plantlets exposed to 300 mM NaCl (58%) as compared to the control plantlets. The plantlets exposed to lower concentration of NaCl (100 mM) recorded a reduction in soluble sugars content (14%) than control plantlets. In comparison to the control, the soluble sugar level in roots increased gradually up to 200 mM NaCl. In the roots of plantlets exposed to 300 mM NaCl there was only less increase in soluble sugar content (<10%). In the roots of the plantlets subjected to higher NaCl concentration (400 mM), there was a reduction (19%) of total soluble sugars content compared to the control plantlets (Fig. 17).

In contrast to NaCl treatment, the CuSO₄ exposure significantly increased the total soluble sugars content in leaves and roots and the increase was gradual with increase in the days of exposure. In leaves, a pronounced increase was found from 10d onwards; whereas in roots it was from 5d. The accumulation in total soluble sugars was 29 and 56% (in leaves) and 65 and 130% (in roots) as compared with the control plantlets on 20d (Fig. 17).

4.7.3 Total soluble protein

The soluble protein content of the leaves and roots increased in response to the NaCl treatment as compared to control plantlets. The

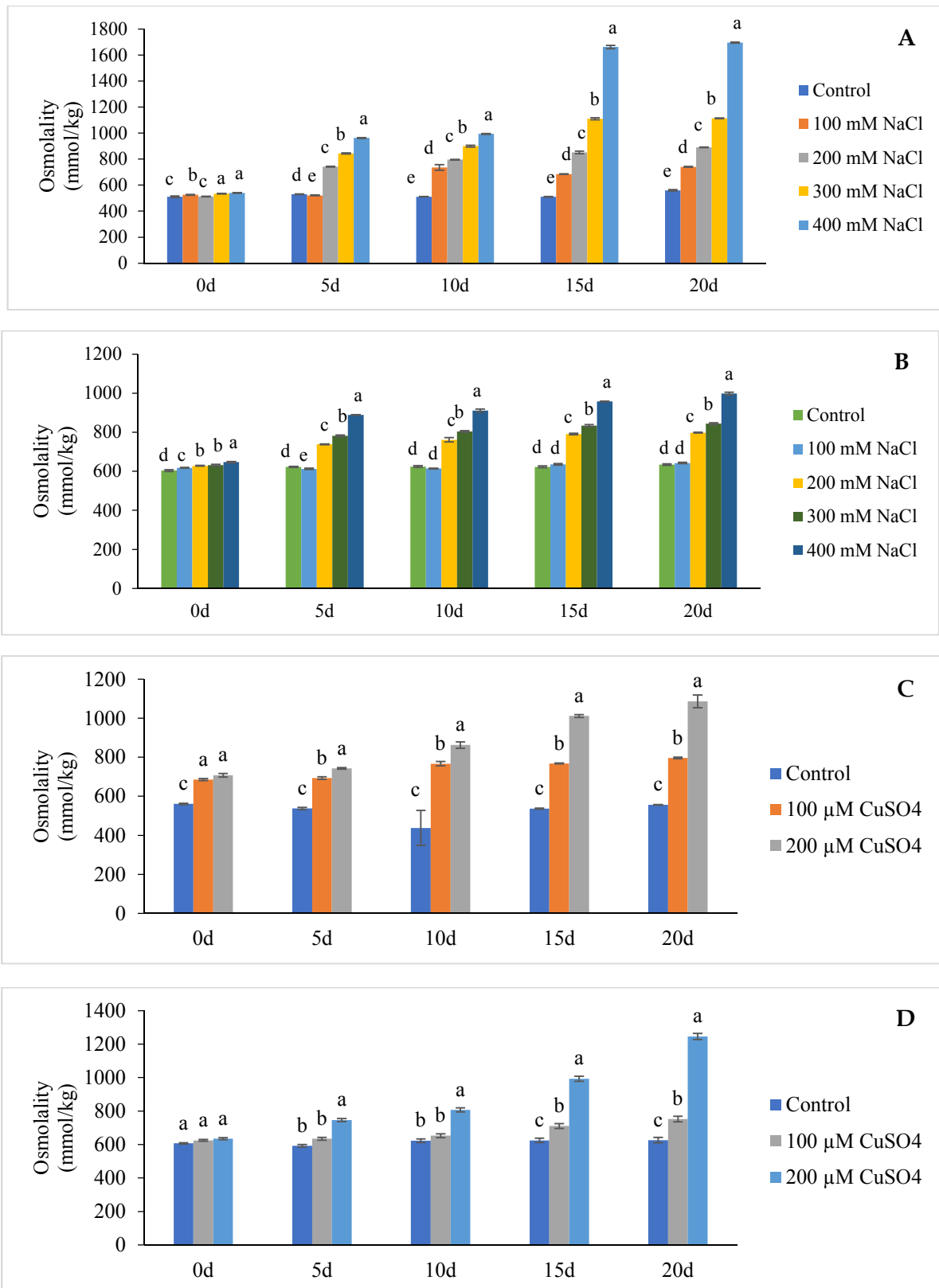


Figure 16. Osmolality in the leaves (A and C) and roots (B and D) of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300 and 400 mM) and CuSO₄ (0, 100 and 200 μM). Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

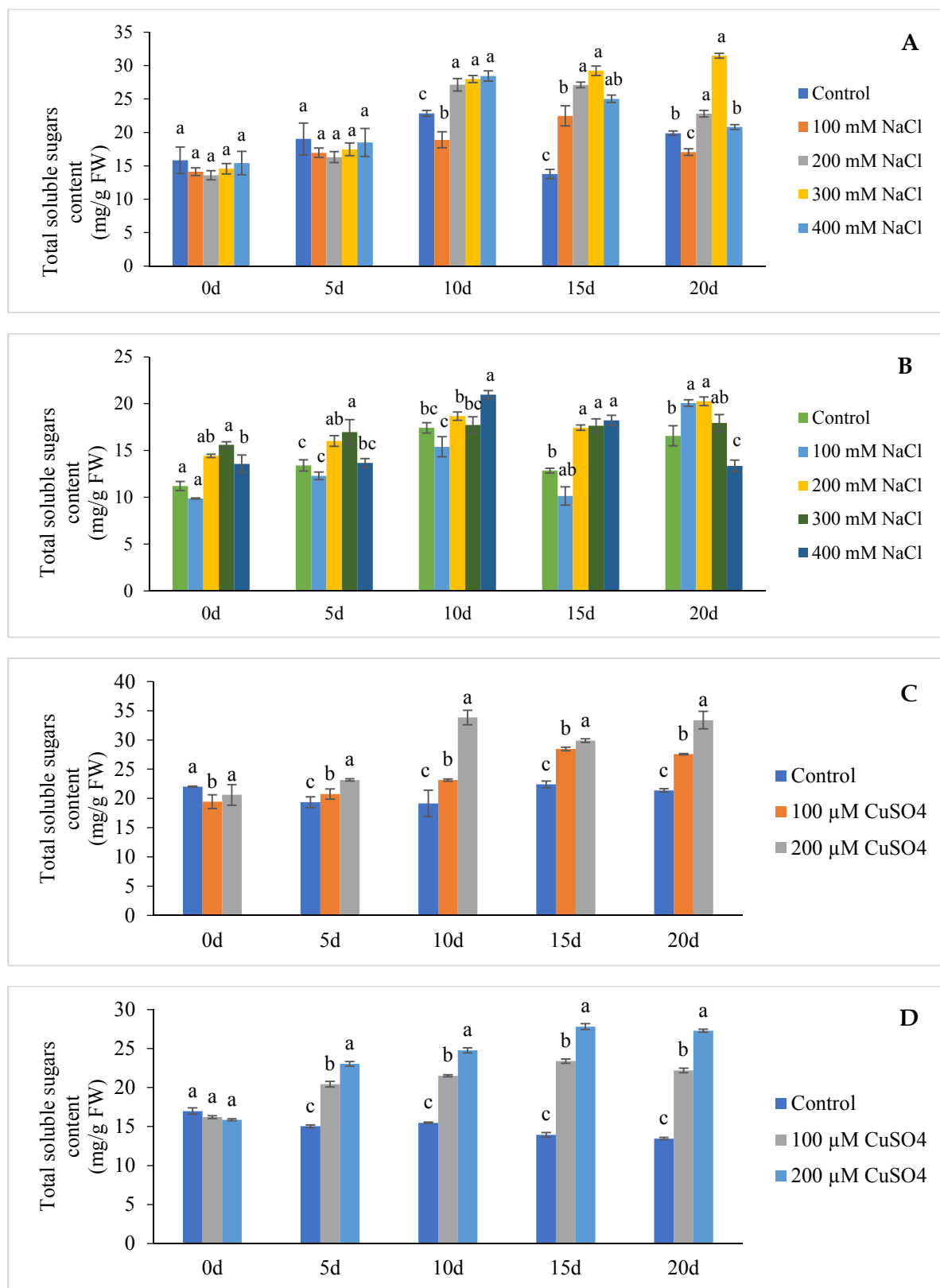


Figure 17. Total soluble sugars content in the leaves (A and C) and roots (B and D) of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300 and 400 mM) and CuSO₄ (0, 100 and 200 μM). Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

increase was more prominent from 10d onwards in both leaves and roots. The higher accumulation of soluble protein was found in the leaves as compared to the roots. The increase was 2.3, 3.5, 4.3 and 5.8-fold in the leaves of the plantlets treated with 100, 200, 300 and 400 NaCl respectively as compared to the control plantlets on 20d. In the case of roots, the total soluble protein content was increased in all treatments except 100 mM NaCl. The increase was 13, 17 and 51% in plantlets treated with 200, 300 and 400 mM NaCl respectively as compared to the control plantlets on 20d. There was a minor decrease in soluble protein content in the plantlets treated with 100 mM NaCl as compared to the control plantlets (Fig. 18).

Similar to the NaCl treatment, CuSO₄ also caused an increase in total soluble protein content in both leaves and roots. The more prominent increase was found in leaves as compared to roots. The increase was 57 and 100% in leaves and 19 and 34% in roots of plantlets subjected to 100 and 200 µM CuSO₄ as compared to the control plantlets on 20d (Fig. 18).

4.7.4 Total free amino acids

The amino acids content of *V. inermis* leaves gradually rose in response to an increase in NaCl content of Hoagland's medium. The plantlets grown in 300 mM NaCl on 20d had the highest level of amino acids, which was 121% higher than the control plantlets. The amino acids content in the roots of *V. inermis* recorded maximum (118%) on 15d of 300 mM NaCl treatment compared with the control plantlets and it was also higher in plantlets grown in the 400 mM NaCl (95%) on 15d (Fig. 19).

The CuSO₄ exposure significantly increased the total free amino acids content in leaves and roots and higher accumulation was found in leaves as compared with roots. The accumulation in total free amino acids in plantlets treated with 100 and 200 µM CuSO₄ was 3 and 4-fold (in leaves) and 52 and

82% (in roots) as compared with the control plantlets on 20d of treatment (Fig. 19).

4.7.5 Proline content

On exposure to NaCl, the proline content in the leaves and roots gradually increased. In comparison to the roots, the increase was greater in the leaves. The increase of the proline content was 4, 14, 15 and 16-fold in leaves of plantlets treated with 100, 200, 300, & 400 mM NaCl respectively. Similar to the leaves, the proline level increased gradually in the roots as well, reaching a maximum (15-fold) in plantlets exposed to 400 mM NaCl (Fig. 20).

There was also increase in proline content of leaves and roots of in plantlets treated with 100 and 200 μ M CuSO₄. The higher accumulation was observed in leaves compared with roots. The plantlets subjected to 100 and 200 μ M CuSO₄ exhibited an increase of 33 and 66% (in leaves) and 12 and 20% (in roots) respectively as compared with control plantlets (Fig. 20).

4.8 Free radical scavenging mechanisms

4.8.1 Non-enzymatic antioxidants

4.8.1.1 Total phenolics content

Exposure of *V. inermis* to NaCl resulted in the augmented level of phenolics in leaves and root tissues. The significant level of total phenolics content was observed from 10d onwards and reached its maximum on 20d. In leaves, the increase was gradual and a pronounced increase (5 and 7-fold) was found in the plantlets treated with 300 and 400 mM NaCl as compared with control plantlets on 20d. The plantlets treated with lower NaCl concentrations (100 and 200 mM) exhibited 1.2 and 2-fold increase in total phenolics content compared with the control plantlets on 20d. In the case of

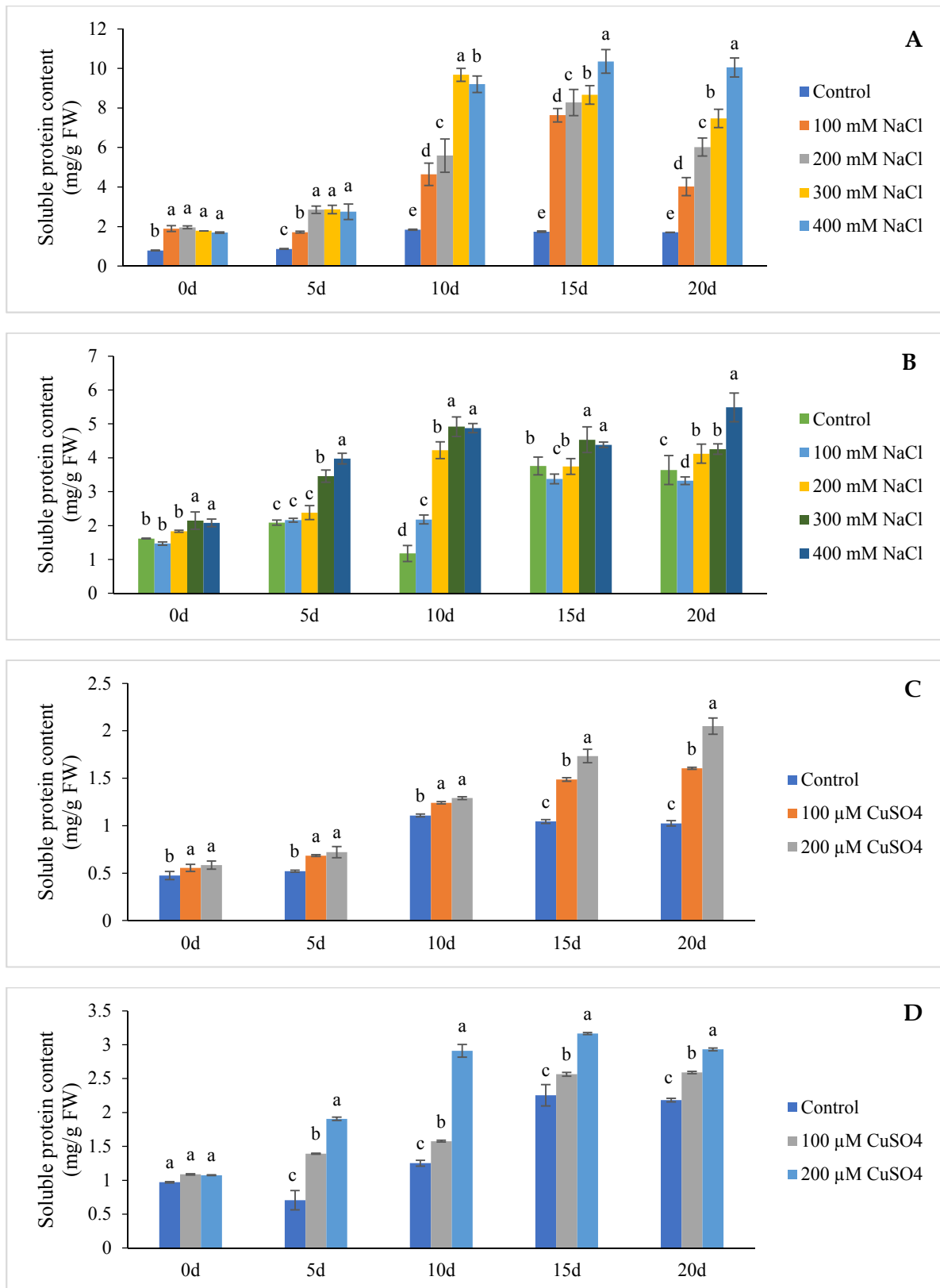


Figure 18. Total soluble protein content in the leaves (A and C) and roots (B and D) of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300 and 400 mM) and CuSO₄ (0, 100 and 200 μ M). Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

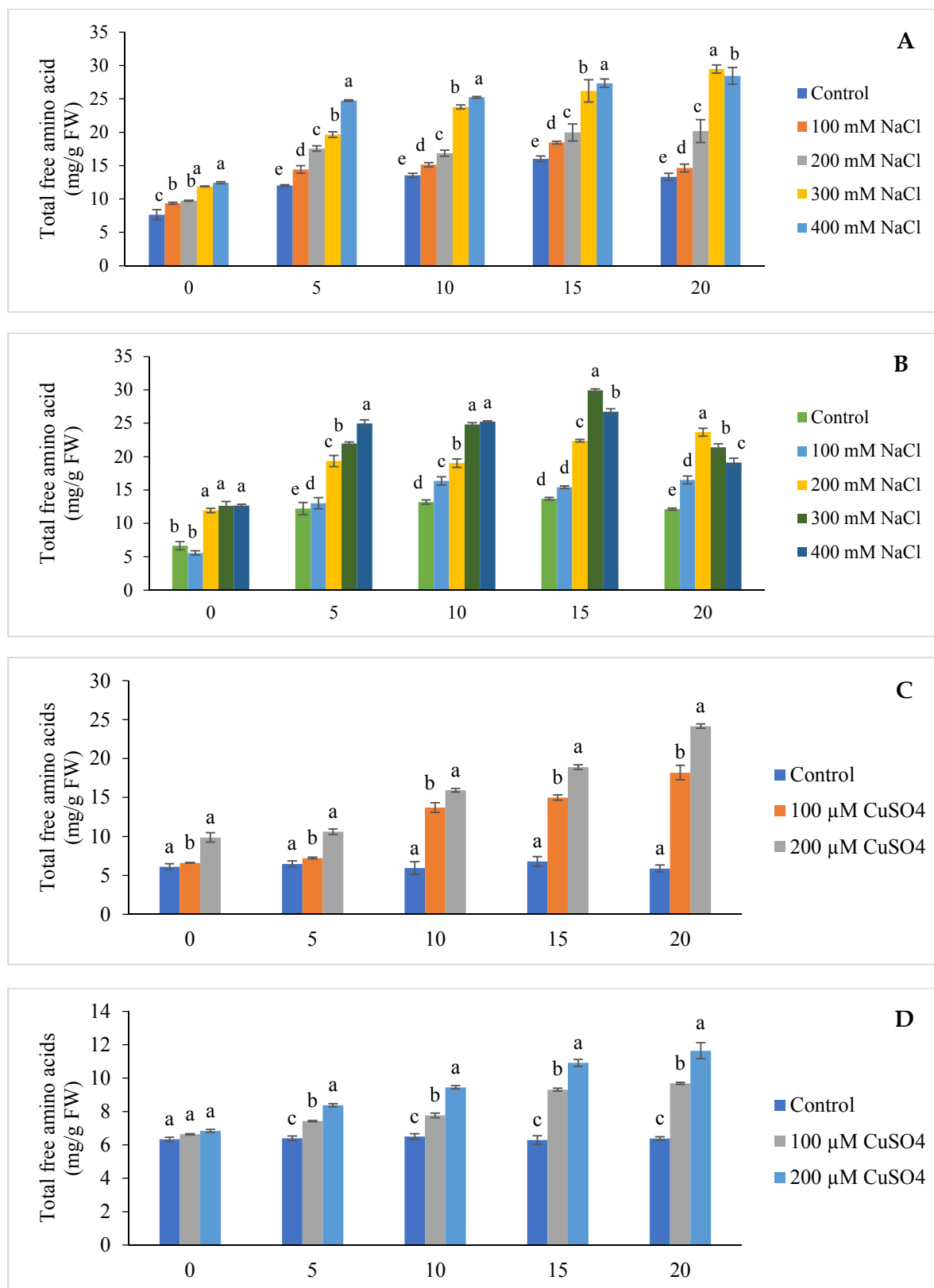


Figure 19. Total free amino acids content in the leaves (A and C) and roots (B and D) of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300 and 400 mM) and CuSO₄ (0, 100 and 200 μM). Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

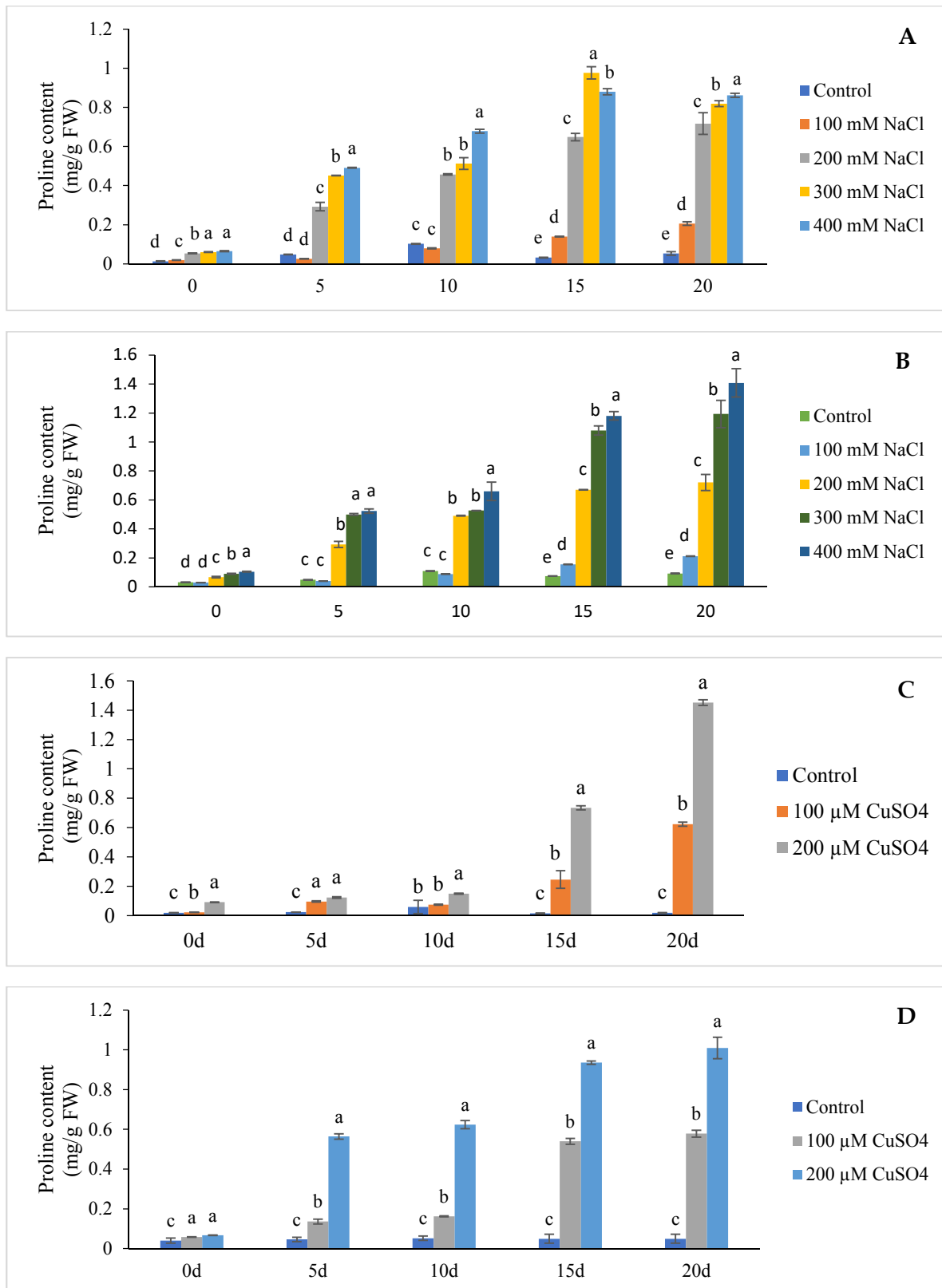


Figure 20. Proline content in the leaves (A and C) and roots (B and D) of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300 and 400 mM) and CuSO₄ (0, 100 and 200 μM). Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

roots, the increase in total phenolics content was 42, 65, 203 and 262% in plantlets treated with 100, 200, 300 and 400 mM NaCl respectively as compared with the control plantlets (Fig. 21).

The plantlets on being subjected to CuSO₄, both leaves and roots recorded significant increase in total phenolics content. The maximum accumulation was found in the leaves compared with that of roots. The increase in total phenolics content was 167 and 187% (in leaves) and 71 and 48% (in roots) in plantlets treated with 100 and 200 µM CuSO₄ on 20d as compared with that of control plantlets (Fig. 21).

4.8.1.2 Ascorbate (AsA) content

V. inermis subjected to increasing NaCl concentrations exhibited a significant increase in ascorbate content of leaves except the plantlets exposed to 400 mM NaCl. The increase was 25, 57 and 49% in the leaves of the plantlets subjected to 100, 200 and 300 mM NaCl respectively as compared with the control plantlets. The higher NaCl did not reduce the ascorbate content and it was maintained as like that of control plantlets. In contrast to this, the root ascorbate content was reduced drastically with increasing NaCl concentration. The maximum reduction (56%) was observed in the roots of the plantlets subjected to 400 mM NaCl as compared with the control plantlets. The lower NaCl concentration had less negative impact (reduction of only 14%) on ascorbate content compared with plantlets grown in other NaCl concentrations (Fig. 22).

There was a gradual increase in ascorbate content in the leaves and roots of *V. inermis* on being subjected to CuSO₄ treatments. The ascorbate content in leaves was gradually increased and exhibited its maximum accumulation on 20d. The increase was 194 and 274% in plantlets subjected to 100 and 200 µM CuSO₄ as compared with that control plantlets. In the

case of roots, the increase in ascorbate content was higher on 15d (53% in 100 and 102% in 200 μM CuSO_4) as compared with control plantlets. On 20d, the increase in ascorbate content was higher than control plantlets but it was less than that recorded on 15d (12% in 100 and 40% 200 μM CuSO_4) (Fig. 22).

4.8.1.3 Glutathione (GSH) content

The plantlets exposed to NaCl recorded an increase in the content of glutathione in leaves and roots. The highest increase in glutathione was observed on 20d. The increase in glutathione content was 17, 20, 40 and 44% in plantlets treated with 100, 200, 300 and 400 mM NaCl respectively as compared with control plantlets. Similar trend of increase was also found in the root glutathione content. The increase in glutathione content was 44, 56, 63 and 66% in plantlets treated with 100, 200, 300 and 400 mM NaCl respectively as compared with control plantlets (Fig. 23).

The CuSO_4 treatment also caused an increment in glutathione content on 20d and it was higher in roots than leaves. The increase in glutathione content of leaves was 42 and 54% in plantlets subjected to 100 and 200 μM CuSO_4 respectively as compared with that control plantlets. Similar to this, glutathione content in roots was also increased in comparison with control plantlets and was 130 and 127% higher in plantlets treated with 100 and 200 CuSO_4 as compared with the control plantlets (Fig. 23).

4.8.1.4 Flavonoids content

Flavonoids content significantly increased on being exposed to increasing NaCl concentrations in both leaves and root tissues of *V. inermis*. The plantlets subjected to 100, 200, 300 and 400 mM NaCl resulted in the augmentation of flavonoid content, to the extent of 2.5 to 3.7-fold in leaves on 20d. Similarly, the root flavonoid content was also increased and it was

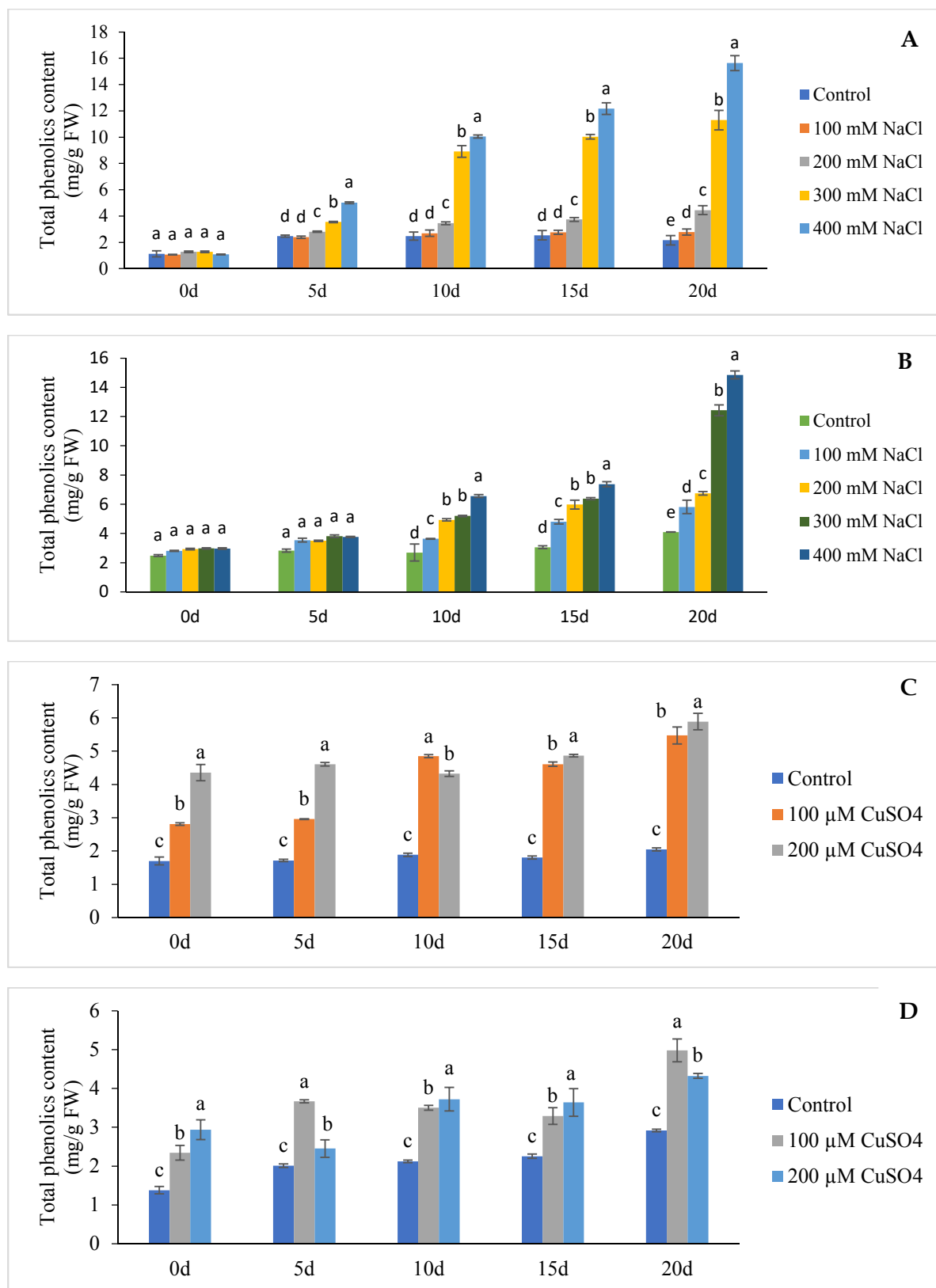


Figure 21. Total phenolics content in the leaves (A and C) and roots (B and D) of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300 and 400 mM) and CuSO₄ (0, 100 and 200 μ M). Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

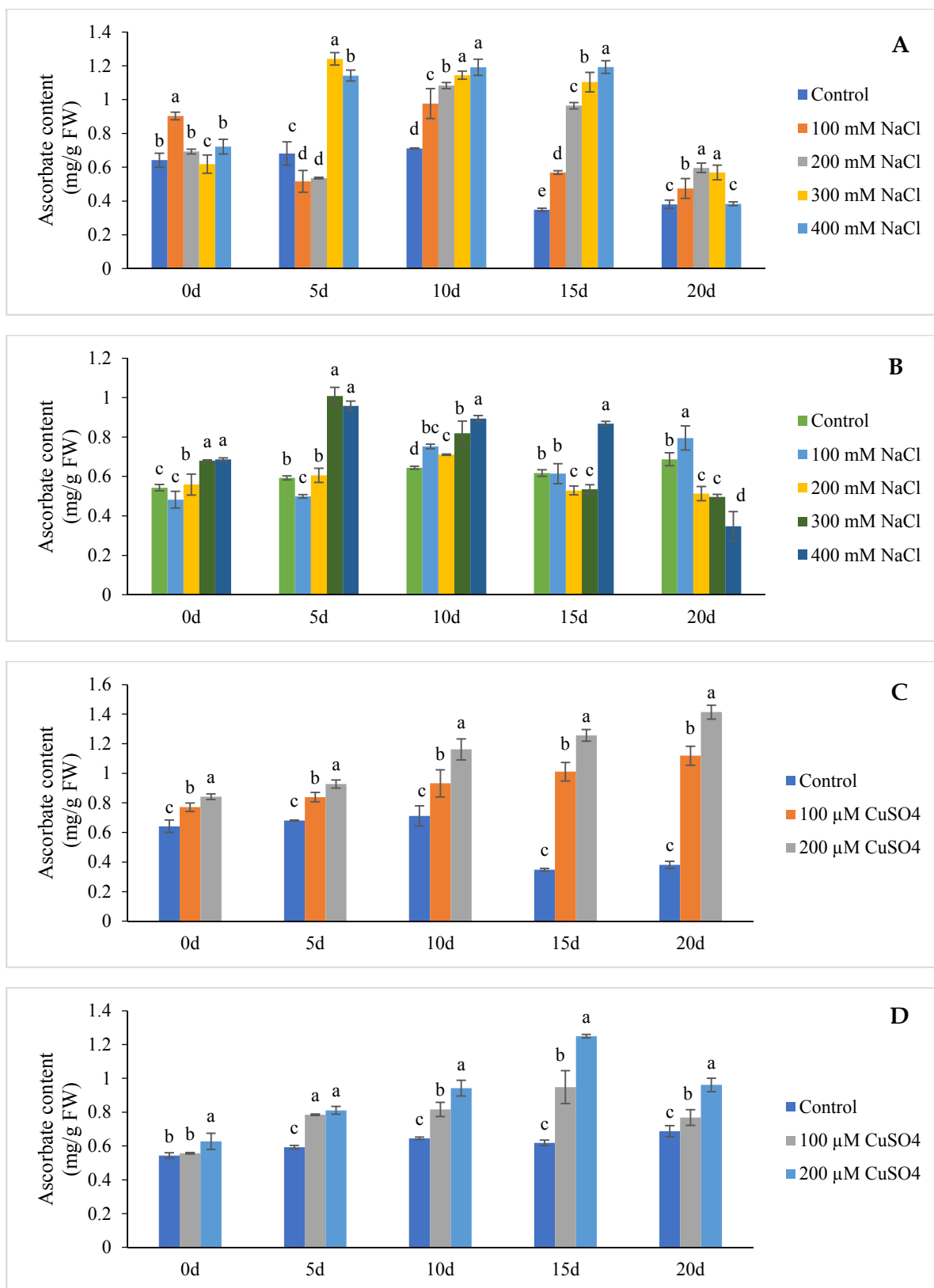


Figure 22. Ascorbate content in the leaves (A and C) and roots (B and D) of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300 and 400 mM) and CuSO₄ (0, 100 and 200 μ M). Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

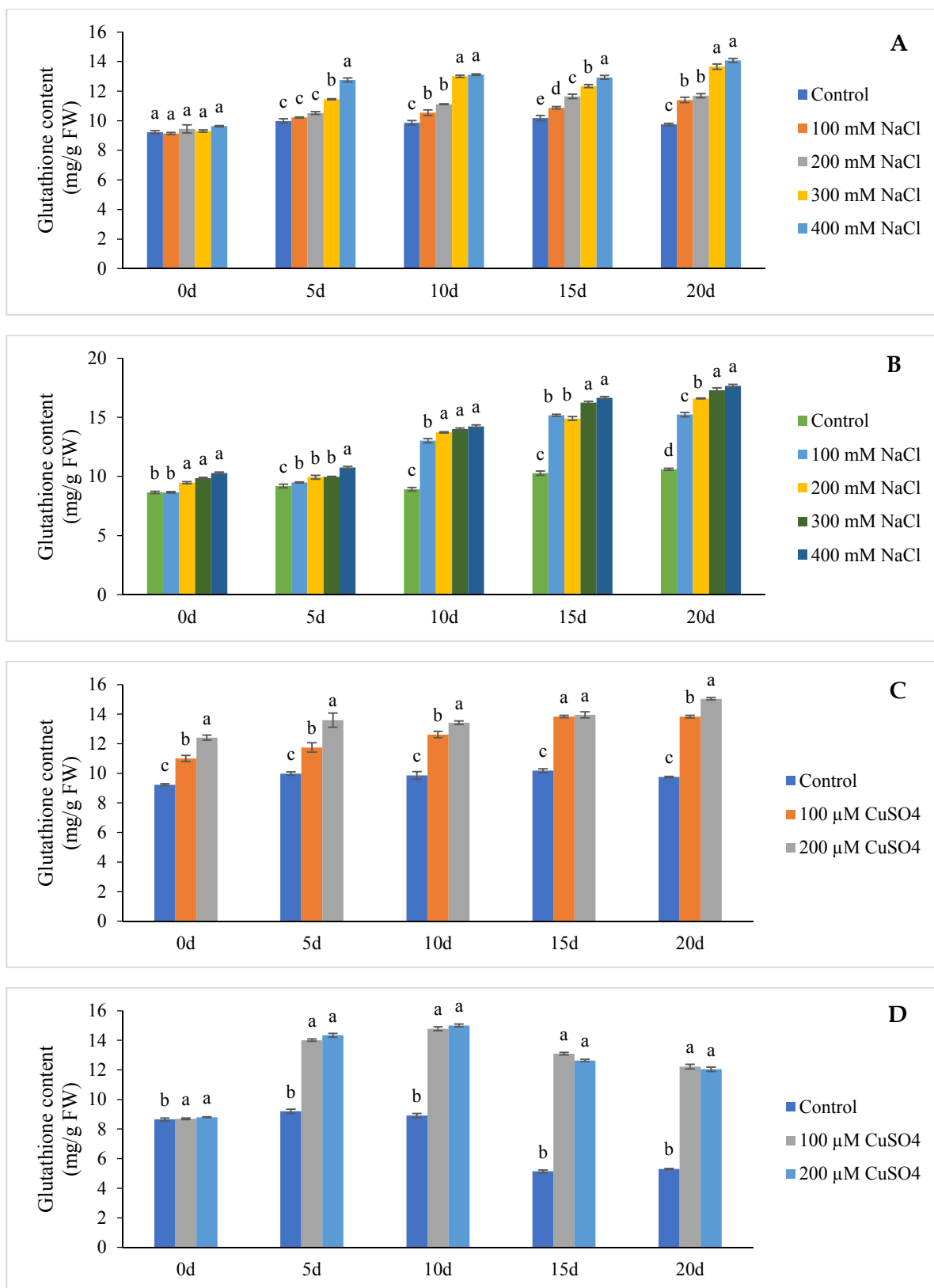


Figure 23. Glutathione content in the leaves (A and C) and roots (B and D) of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300 and 400 mM) and CuSO₄ (0, 100 and 200 μ M). Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

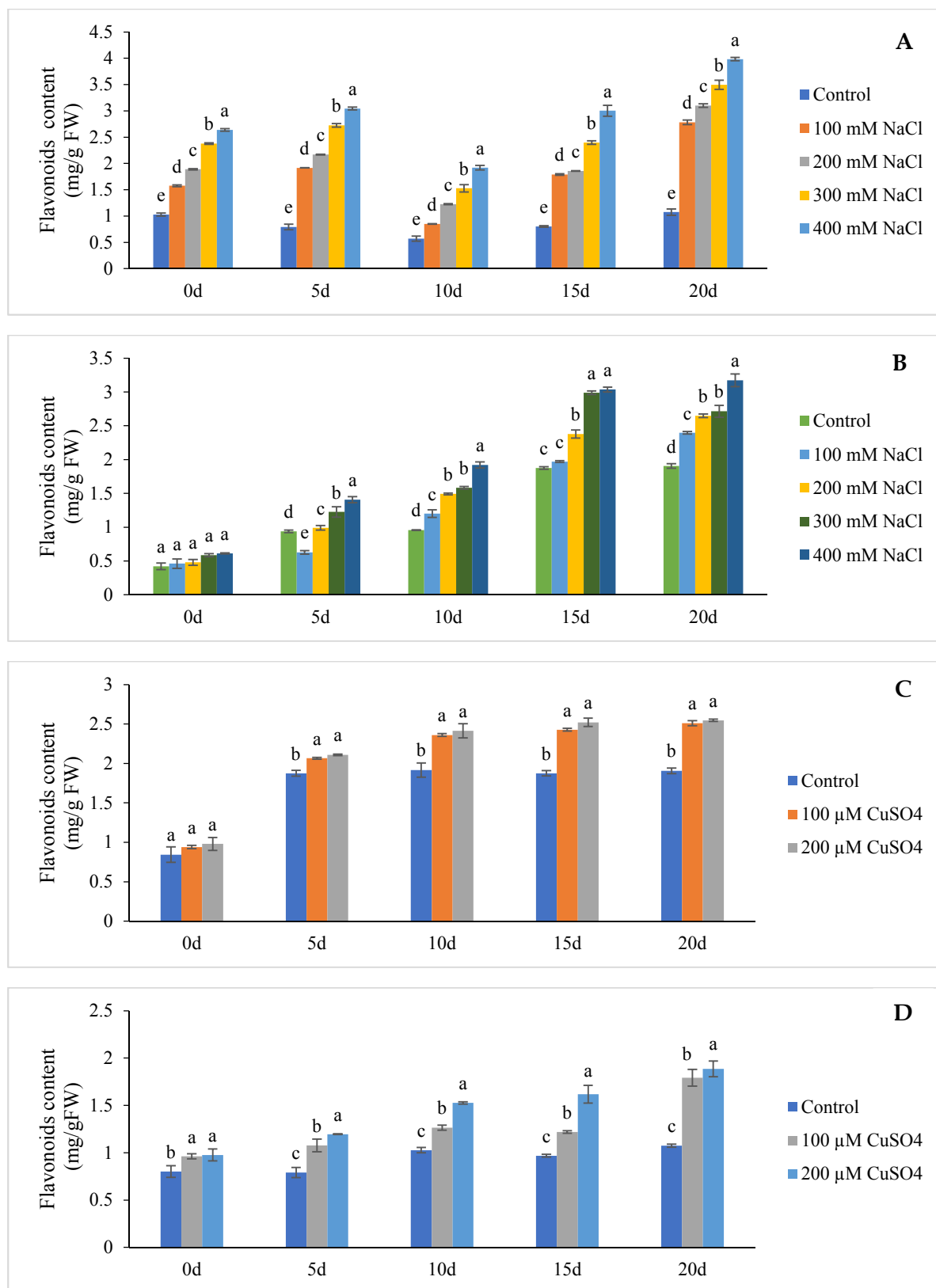


Figure 24. Flavonoids content in the leaves (A and C) and roots (B and D) of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300 and 400 mM) and CuSO₄ (0, 100 and 200 μ M). Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

26, 39, 42 and 66% higher in plantlets subjected to 100, 200, 300 and 400 mM NaCl respectively as compared with control plantlets (Fig. 24).

The CuSO₄ exposure positively influenced the flavonoid biosynthesis and the increase was maximum in higher CuSO₄ treatment (200 µM). The higher flavonoid content was found in roots than leaves. The accumulation was 32 and 34% (in leaves) and 2.6 and 2.7 -fold (in roots) in plantlets subjected to 100 and 200 µM CuSO₄ as compared with the control plantlets (Fig. 24).

4.8.2 Enzymatic antioxidants

4.8.2.1 Superoxide dismutase (SOD)

The activity of SOD exhibits an enhancement in leaves and roots of *V. inermis* subjected to NaCl as compared with the control plantlets. In leaves, the maximum SOD activity was found on 15d of treatment and the increase was 17, 87, 209 and 199% in plantlets treated with 100, 200, 300 and 400 mM NaCl respectively as compared with control plantlets. On 20d of treatment, there was slight reduction in SOD activity as compared with the activity on 15d. In the case of roots, similar observations were found. The maximum SOD activity was found on 15d and the increase was 20, 60, 86 and 102% in plantlets treated with 100, 200, 300 and 400 mM NaCl respectively as compared with control plantlets (Fig. 25).

The SOD activity in the plantlets exposed to CuSO₄ was also increased upon increasing the CuSO₄ concentration in the medium. In leaves, the higher activity was observed in plantlets treated with 200 µM on 20d. The increase was 2.5-fold higher than that of control plantlets. The plantlets grown in lower CuSO₄ concentration also showed an increase and was only 1.9-fold as compared with control plants. In the case of roots, the activity was increased in all the days of treatment and among them, the higher

activity was observed on 10d of treatment (4-fold in 100 μ M and 1.5-fold in 200 μ M) (Fig. 25).

4.8.2.2 Guaiacol peroxidase (POD)

The POD activity of the leaves exhibits progressive increase with respect to NaCl concentration, with the exception of reduced activity in plantlets treated with 100 mM NaCl. The highest activity in POD was observed on 20d of treatment. However, the POD activity increased significantly in plantlets exposed to 200, 300 and 400 mM NaCl and the increase in POD activity was 46, 133 and 189% respectively as compared with control plantlets (Fig. 26).

In the case of roots, highest activity was overserved on 10d of treatment and it was in the range of 2 to 3.6-fold as compared to control plantlets. The lower NaCl concentration (100 mM) treatment caused reduction in POD activity and the reduction was 22 and 35% on 10 and 20d respectively as compared with control plantlets. On 20d of treatment, POD activity was slightly reduced than 10d. The increase in POD activity was 28, 98 and 141% in plantlets exposed to 200, 300 and 400 mM NaCl respectively as compared with control plantlets (Fig. 26).

Exposure to CuSO₄ increased the activity of POD in the leaves and roots of *V. inermis* and the highest activity was observed on 20d of treatment. The increase in POD activity was 24 and 103% (leaves) and 110 and 193% (roots) in plantlets subjected to 100 and 200 μ M CuSO₄ respectively as compared with the control plantlets (Fig. 26).

4.8.2.3 Ascorbate peroxidase (APX)

The APX activity was increased in response to NaCl treatment. The higher increase was observed in the leaves than roots on 20d. In the case of

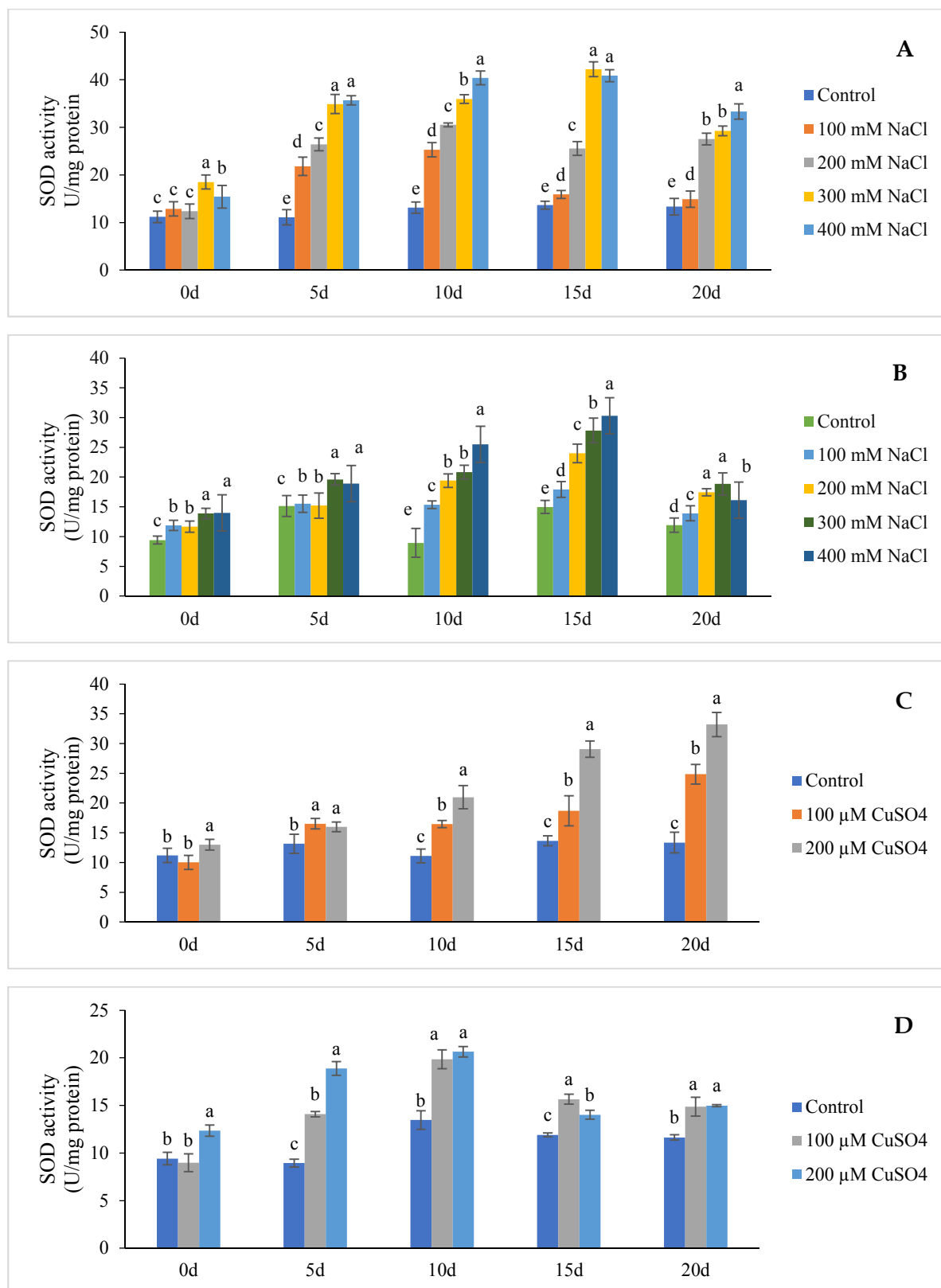


Figure 25. Superoxide dismutase (SOD) activity in leaves (A) and roots (B) of NaCl treated and leaves (C) and roots (D) of CuSO₄ treated *V. inermis* plantlets. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

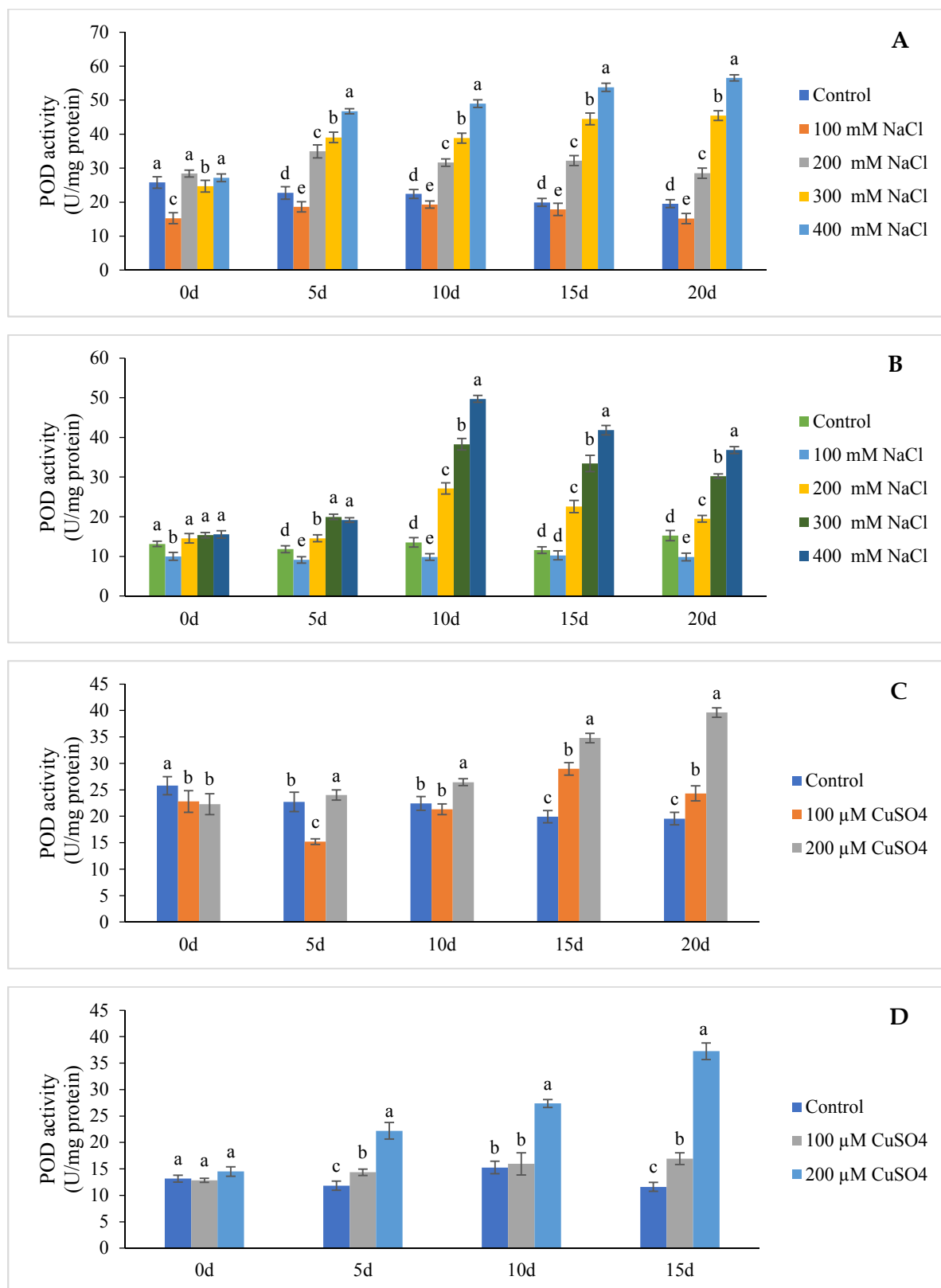


Figure 26. Guaiacol peroxidase (POD) activity in leaves (A) and roots (B) of NaCl treated and leaves (C) and roots (D) of CuSO₄ treated *V. inermis* plantlets. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

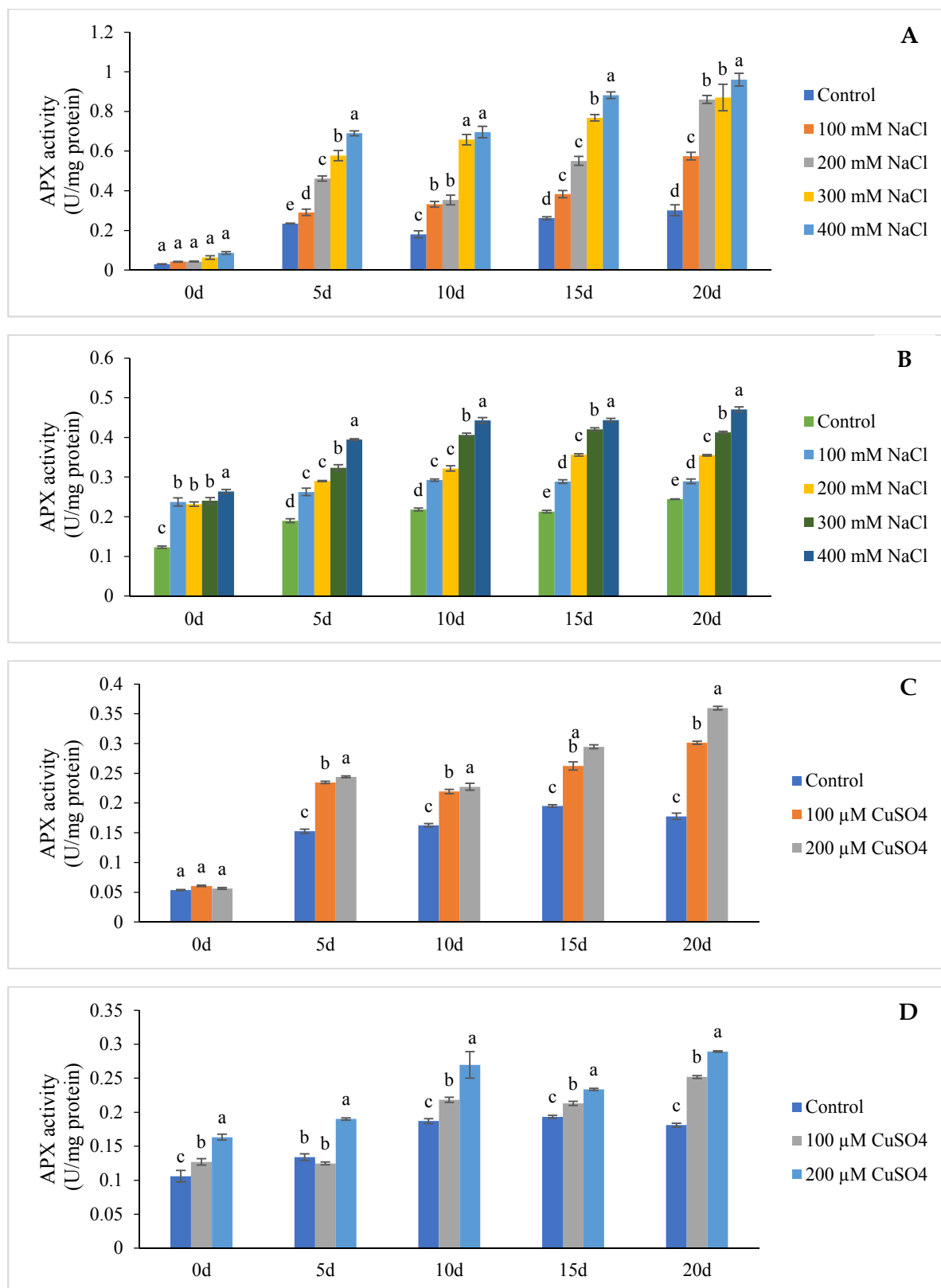


Figure 27. Ascorbate peroxidase (APX) activity in leaves (A) and roots (B) of NaCl treated and leaves (C) and roots (D) of CuSO₄ treated *V. inermis* plantlets. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

leaves, the increase was 1.9, 2.85, 2.88 and 3.18-fold in plantlets subjected to 200, 300 and 400 mM NaCl respectively as compared with control plantlets. At the same time the increase in root APX activity was only 18, 45, 69, and 93% in plantlets subjected to 200, 300 and 400 mM NaCl respectively as compared with control plantlets (Fig. 27).

The CuSO₄ exposure caused an increase in APX activity and the highest activity was in leaves than roots on 20d. In the case of leaves, the APX activity was increased upto 102% than control plantlets. The root APX activity was 39 and 60% in plantlets subjected to 100 and 200 µM CuSO₄ respectively as compared with the control plantlets (Fig. 27).

4.9 Bioaccumulation and translocation of Na and Cu

The bioaccumulation of Na and Cu in different plant parts such as leaf, stem and root were determined. The bioaccumulation of Na and Cu in *V. inermis* plantlets was increased gradually with increasing concentration of NaCl and CuSO₄ in the medium. Among the different plant parts, leaf showed higher accumulation of Na as compared with stem and root. In leaf 63.05±3.152 mg/g DW of Na was accumulated on exposure to 400 mM NaCl, while in control plantlets it was only 36.10±1.80 mg/g DW. In the case of root tissues, the accumulation was higher than control plantlets and it was 17.44±0.872 mg/g DW in 400 mM NaCl. When comparing the percentage of increase in root and leaf, the roots showed higher accumulation percentage. The percentage of increase was 559 and 75% in root and leaf tissues of plantlets exposed to 400 mM NaCl as compared with their control plantlets. The stem of control plantlets accumulates only 1.44±0.072 mg/g DW of Na, but it was enhanced upto 6.56±0.328 mg/g DW in plantlets exposed to 400 mM NaCl (Table 13).

The translocation factor (TF) determine the ability of translocation of Na/Cu from root to upper part of the plant. The TF decreased in plantlets exposed to NaCl and maximum reduction was recorded in plantlets exposed to 300 and 400 mM NaCl. In the plants subjected to 300 and 400 mM NaCl, TF was 3.92 ± 0.196 and 3.99 ± 0.199 respectively. The higher TF was observed in control plantlets (14.21 ± 0.710) as compared with the other treatments (Table 13).

With respect to bioaccumulation of Cu in *V. inermis* plantlets exposed to different concentrations of CuSO_4 , maximum accumulation was observed in plantlets treated with 200 μM CuSO_4 . Among the different plant parts studied, maximum accumulation was found in root tissues and it also showed a progressive increase with the increase in concentration of CuSO_4 in the medium. The root tissues of the plantlets treated with 200 μM CuSO_4 accumulated 5144.04 ± 257.2 $\mu\text{g/g}$ DW of Cu at the same time control root accumulated only 5.54 ± 0.277 $\mu\text{g/g}$ DW. Next to roots, higher rate of Cu accumulation was observed in the stem of *V. inermis*. The stem of the plantlets exposed to 200 μM CuSO_4 accumulated 469.55 ± 23.47 $\mu\text{g/g}$ DW of Cu. In the case of the leaves, greater Cu accumulation was seen in plantlets subjected to 200 mM CuSO_4 and the value was 26.15 ± 1.307 $\mu\text{g/g}$ DW compared to control leaves where the Cu accumulation was only 3.07 ± 0.153 $\mu\text{g/g}$ DW (Table 13).

When compared with the TF of NaCl treated plantlets, the TF of the CuSO_4 treated plantlets were very low and it was even below 1 (0.096 ± 0.004) in plantlets exposed to higher CuSO_4 treatment. Another interesting factor was that, the treatment with mild concentration (100 μM), resulted in higher TF (1.59 ± 0.079) and it was higher than that of control plantlets (Table 13).

Table 13. Bioaccumulation of sodium (Na) and copper (Cu) and translocation factor (TF) in the leaf, stem and root of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300 and 400 mM) and CuSO₄ (0, 100 and 200 µM). Values are the mean ±SE of three different experiments. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

NaCl Treatment (mM)	Na content (mg/g DW)			TF
	Leaf	Stem	Root	
0	36.10±1.805 ^c	1.44±0.072 ^e	2.64±0.132 ^e	14.21±0.711 ^a
100	23.63±1.181 ^e	2.89±0.144 ^d	5.01±0.250 ^d	5.29±0.264 ^b
200	29.31±1.465 ^d	4.08±0.204 ^c	9.85±0.492 ^c	3.38±0.169 ^d
300	41.95±1.897 ^b	6.12±0.295 ^b	12.25±0.712 ^b	3.92±0.196 ^c
400	63.05±3.152 ^a	6.56±0.328 ^a	17.44±0.872 ^a	3.99±0.199 ^c
CuSO ₄ Treatment (µM)	Cu (µg/ g DW)			TF
	Leaf	Stem	Root	
0	3.07±0.153 ^c	3.95±0.197 ^c	5.54±0.277 ^c	1.26±0.063 ^b
100	12.91±0.645 ^b	251.99±12.5 ^b	166.60±8.33 ^b	1.59±0.079 ^a
200	26.15±1.307 ^a	469.55±23.4 ^a	5144.04±257 ^a	0.096±0.004 ^c

4.10 Subcellular distribution

The subcellular localization of Na, Cu, K and Ca was analysed in the control as well as NaCl and CuSO₄ treated samples of *V. inermis*. Various subcellular fractions like cell wall, chloroplast, nucleus, mitochondria, ribosomes, vacuole and cytoplasm were critically analysed to detect the presence of Na, Cu, K and Ca. Various subcellular fractions contained the following cellular components, fraction I; the cell wall and cell wall debris, fraction II; chloroplasts and cell nuclei, fraction III; mitochondria, fraction IV; ribosomes, vacuoles and cytoplasm.

4.10.1 NaCl treated plantlets

The Na content was remarkably higher in the soluble fraction of leaf tissue containing ribosomes, vacuoles and cytoplasm, i.e., 65-fold increase over the control leaves. However, the same fraction of the root and stem had only 4 and 3-fold increase in Na content as compared with the control plantlets. Similar trend of increase was observed in cell wall and wall debris fraction. The accumulation of Na was 253, 280 and 303% in leaf, root and stem respectively as compared with the control plantlets. On the other hand, in the mitochondrial fraction, Na content decreased significantly in all the tissues, i.e., 24, 62 and 12% reduction in the leaf, root and stem tissues respectively as compared with the control plantlets (Table 14).

In general, the content of K was decreased in the fractions of cell wall and cell wall debris, chloroplast, nuclei and mitochondria of leaf and root tissues. However, a sharp increase in K content was found in cell wall and cell wall debris fraction of stem tissue, followed by chloroplast and cell nuclei fraction and mitochondrial fraction. In the stem tissue, the K content in fraction containing ribosomes, vacuoles and cytoplasm decreased

significantly and it was 29% lesser as compared to the control plantlets (Table 14).

Ca content decreased in all the fractions of root tissues analysed, i.e., 56, 60, 20 and 76% reduction were recorded in the fractions I, II, III and IV respectively. Interestingly, a sharp enhancement in the Ca content was recorded in the fraction containing ribosomes, vacuoles and cytoplasm of leaf tissue, i.e., 40-fold increase over the control leaves (Table 14).

In general Cu content decreased in all the subcellular fractions of the plant tissues and an exception was found in fraction I and fraction IV of leaf and root. Higher reduction was observed in the chloroplast and nuclei fraction of leaf, root and stem and it was 97, 96 and 87% respectively as compared with the control plantlets (Table 14).

4.10.2 CuSO₄ treated plantlets

In CuSO₄ treated plantlets, the cell wall and cell wall debris fraction of the leaf, root and stem showed higher accumulation of Cu. Among them the highest accumulation was in the cell wall and wall debris of root tissue. It was 399% higher than that of control plantlets. The soluble fraction (fraction IV) also higher showed accumulation. The root and leaf soluble fraction of ribosomes, vacuole and cytoplasm recorded 5 and 18-fold increase respectively in the Cu accumulation over control plantlets (Table 15).

The subcellular distribution of Na in CuSO₄ treated plantlets exhibited higher accumulation in fraction IV. Most of the Na ions absorbed from the medium were restricted within the vacuole, cytoplasm and ribosomes. The other fractions, containing cell wall and cell wall debris, chloroplast and nuclei, and mitochondria had variable level of Na as compared with the control plantlets (Table 15).

Table 14. Subcellular localization of Na, K, Ca and Cu in *V. inermis* plantlets (leaves, roots, stem) subjected to 400 mM NaCl. Values are the means $\pm$ SE of the three different experiments. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

Plant Parts	Subcellular Fraction	Control				400 mM NaCl			
		Na ($\mu\text{g/g FW}$)	K ($\mu\text{g/g FW}$)	Ca ($\mu\text{g/g FW}$)	Cu ($\mu\text{g/g FW}$)	Na ($\mu\text{g/g FW}$)	K ($\mu\text{g/g FW}$)	Ca ($\mu\text{g/g FW}$)	Cu ($\mu\text{g/g FW}$)
Leaf	Fraction I	277.05 $\pm$ 13.85 ^a	282.71 $\pm$ 14.13 ^a	5.979 $\pm$ 0.298 ^a	0.652 $\pm$ 0.032 ^b	977.94 $\pm$ 48.89 ^b	268.02 $\pm$ 13.4 ^b	10.539 $\pm$ 0.05 ^a	0.712 $\pm$ 0.035 ^b
	Fraction II	144.66 $\pm$ 7.23 ^c	105.01 $\pm$ 5.25 ^b	2.875 $\pm$ 0.143 ^b	0.698 $\pm$ 0.034 ^b	197.36 $\pm$ 9.868 ^c	31.65 $\pm$ 1.58 ^c	2.099 $\pm$ 0.104 ^c	0.02 $\pm$ 0.001 ^d
	Fraction III	220.17 $\pm$ 11.008 ^b	44.73 $\pm$ 2.23 ^c	1.811 $\pm$ 0.090 ^c	1.042 $\pm$ 0.052 ^a	167.54 $\pm$ 8.377 ^d	14.99 $\pm$ 0.749 ^d	0.828 $\pm$ 0.041 ^d	1.28 $\pm$ 0.064 ^a
	Fraction IV	32.901 $\pm$ 1.64 ^d	38.84 $\pm$ 1.94 ^d	0.225 $\pm$ 0.011 ^d	0.135 $\pm$ 0.006 ^c	2133.34 $\pm$ 106.6 ^a	614.49 $\pm$ 30.72 ^a	9.174 $\pm$ 0.458 ^b	0.371 $\pm$ 0.018 ^c
Root	Fraction I	145.33 $\pm$ 7.266 ^c	638.15 $\pm$ 31.90	7.919 $\pm$ 0.395 ^a	1.858 $\pm$ 0.092 ^a	551.93 $\pm$ 27.59 ^b	349.40 $\pm$ 17.47 ^b	3.495 $\pm$ 0.174 ^a	2.943 $\pm$ 0.147 ^a
	Fraction II	76.60 $\pm$ 3.83 ^d	46.14 $\pm$ 2.30	1.898 $\pm$ 0.094 ^c	0.924 $\pm$ 0.046 ^b	83.16 $\pm$ 4.15 ^d	12.96 $\pm$ 0.648 ^d	0.75 $\pm$ 0.037 ^d	0.0381 $\pm$ 0.001 ^d
	Fraction III	305.66 $\pm$ 15.28 ^a	33.93 $\pm$ 1.69	1.260 $\pm$ 0.063 ^c	1.879 $\pm$ 0.093 ^a	116.39 $\pm$ 5.81 ^c	15.86 $\pm$ 0.793 ^c	1.010 $\pm$ 0.05 ^b	0.289 $\pm$ 0.014 ^c
	Fraction IV	252.55 $\pm$ 12.62 ^b	2051.08 $\pm$ 102.5	3.911 $\pm$ 0.195 ^b	0.805 $\pm$ 0.040 ^c	951.78 $\pm$ 47.589 ^a	639.94 $\pm$ 31.99 ^a	0.920 $\pm$ 0.046 ^c	0.901 $\pm$ 0.045 ^b
Stem	Fraction I	263.15 $\pm$ 13.15 ^a	40.33 $\pm$ 2.01	9.054 $\pm$ 0.452 ^a	1.710 $\pm$ 0.085 ^b	1061.51 $\pm$ 53.07 ^a	1061.51 $\pm$ 53.07 ^a	19.357 $\pm$ 0.967 ^a	1.474 $\pm$ 0.073 ^a
	Fraction II	76.97 $\pm$ 3.84 ^d	40.33 $\pm$ 2.01	1.356 $\pm$ 0.067 ^b	0.781 $\pm$ 0.039 ^c	134.35 $\pm$ 6.71 ^c	40.43 $\pm$ 2.02 ^c	2.294 $\pm$ 0.114 ^c	0.101 $\pm$ 0.005 ^d
	Fraction III	108.41 $\pm$ 5.42 ^c	16.65 $\pm$ 0.83	1.190 $\pm$ 0.05 ^b	1.736 $\pm$ 0.086 ^b	94.97 $\pm$ 4.74 ^d	23.54 $\pm$ 1.17 ^d	0.617 $\pm$ 0.03 ^d	0.319 $\pm$ 0.01 ^c
	Fraction IV	232.70 $\pm$ 11.63 ^b	880.99 $\pm$ 44.04	9.174 $\pm$ 0.458 ^a	6.399 $\pm$ 0.319 ^a	714.62 $\pm$ 35.73 ^b	627.45 $\pm$ 31.37 ^b	3.01 $\pm$ 0.15 ^b	0.543 $\pm$ 0.027 ^b

Table 15. Subcellular localization of Na, K, Ca and Cu in *V. inermis* plantlets (leaves, roots, stem) subjected to 200 μ M CuSO₄. Values are the means $\pm$ SE of the three different experiments. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

Plant Parts	Subcellular Fraction	Control				200 μ M CuSO ₄			
		Na (μ g/g FW)	K (μ g/g FW)	Ca (μ g/g FW)	Cu (μ g/g FW)	Na (μ g/g FW)	K (μ g/g FW)	Ca (μ g/g FW)	Cu (μ g/g FW)
Leaf	Fraction I	277.05 $\pm$ 13.85 ^a	282.71 $\pm$ 14.13 ^a	5.979 $\pm$ 0.298 ^a	0.652 $\pm$ 0.032 ^b	654.404 $\pm$ 32.720 ^b	363.726 $\pm$ 18.186 ^b	5.252 $\pm$ 0.262 ^a	1.780 $\pm$ 0.089 ^b
	Fraction II	144.66 $\pm$ 7.23 ^c	105.01 $\pm$ 5.25 ^b	2.875 $\pm$ 0.143 ^b	0.698 $\pm$ 0.034 ^b	155.725 $\pm$ 7.786 ^d	33.752 $\pm$ 1.687 ^d	2.036 $\pm$ 0.101 ^b	0.512 $\pm$ 0.025 ^c
	Fraction III	220.17 $\pm$ 11.008 ^b	44.73 $\pm$ 2.23 ^c	1.811 $\pm$ 0.090 ^c	1.042 $\pm$ 0.052 ^a	202.357 $\pm$ 10.117 ^c	71.329 $\pm$ 3.566 ^c	1.767 $\pm$ 0.088 ^c	0.418 $\pm$ 0.021 ^c
	Fraction IV	32.901 $\pm$ 1.64 ^d	38.84 $\pm$ 1.94 ^d	0.225 $\pm$ 0.011 ^d	0.135 $\pm$ 0.006 ^c	1059.803 $\pm$ 52.99 ^a	948.154 $\pm$ 47.407 ^a	2.976 $\pm$ 0.1488 ^b	2.399 $\pm$ 0.119 ^a
Root	Fraction I	145.33 $\pm$ 7.266 ^c	638.15 $\pm$ 31.90	7.919 $\pm$ 0.395 ^a	1.710 $\pm$ 0.085 ^a	481.403 $\pm$ 24.07 ^b	180.479 $\pm$ 9.023 ^b	8.372 $\pm$ 0.4186 ^a	8.530 $\pm$ 0.426 ^a
	Fraction II	76.60 $\pm$ 3.83 ^d	46.14 $\pm$ 2.30	1.898 $\pm$ 0.094 ^c	0.924 $\pm$ 0.046 ^b	355.020 $\pm$ 17.751 ^c	73.595 $\pm$ 3.679 ^c	4.901 $\pm$ 0.245 ^b	4.901 $\pm$ 0.245 ^b
	Fraction III	305.66 $\pm$ 15.28 ^a	33.93 $\pm$ 1.69	1.260 $\pm$ 0.063 ^c	1.879 $\pm$ 0.093 ^a	246.861 $\pm$ 12.343 ^d	52.731 $\pm$ 2.636 ^d	3.581 $\pm$ 0.179 ^c	3.581 $\pm$ 0.179 ^c
	Fraction IV	252.55 $\pm$ 12.62 ^b	2051.08 $\pm$ 102.5	3.911 $\pm$ 0.195 ^b	0.805 $\pm$ 0.040 ^c	637.608 $\pm$ 31.88 ^a	1189.737 $\pm$ 59.486 ^a	4.071 $\pm$ 0.203 ^b	4.071 $\pm$ 0.203 ^b

Plant Parts	Subcellular Fraction	Control				200 μ M CuSO ₄			
		Na (μ g/g FW)	K (μ g/g FW)	Ca (μ g/g FW)	Cu (μ g/g FW)	Na (μ g/g FW)	K (μ g/g FW)	Ca (μ g/g FW)	Cu (μ g/g FW)
Stem	Fraction I	263.15 $\pm$ 13.15 ^a	40.33 $\pm$ 2.01	9.054 $\pm$ 0.452 ^a	1.858 $\pm$ 0.092 ^b	1631.606 $\pm$ 81.580 ^b	466.539 $\pm$ 23.326 ^b	14.853 $\pm$ 0.742 ^a	8.372 $\pm$ 0.418 ^a
	Fraction II	76.97 $\pm$ 3.84 ^d	40.33 $\pm$ 2.01	1.356 $\pm$ 0.067 ^b	0.781 $\pm$ 0.039 ^c	364.447 $\pm$ 18.222 ^d	72.494 $\pm$ 3.624 ^d	4.586 $\pm$ 0.229 ^c	1.274 $\pm$ 0.063 ^c
	Fraction III	108.41 $\pm$ 5.42 ^c	16.65 $\pm$ 0.83	1.190 $\pm$ 0.05 ^b	1.736 $\pm$ 0.086 ^b	508.864 $\pm$ 25.443 ^c	95.570 $\pm$ 4.778 ^c	5.120 $\pm$ 0.256 ^b	1.025 $\pm$ 0.051 ^c
	Fraction IV	232.70 $\pm$ 11.63 ^b	880.99 $\pm$ 44.04	9.174 $\pm$ 0.458 ^a	6.399 $\pm$ 0.319 ^a	2021.019 $\pm$ 101.050 ^a	802.949 $\pm$ 40.147 ^a	5.717 $\pm$ 0.285 ^b	2.343 $\pm$ 0.117 ^b

The K content was decreased in cell wall and wall debris fraction and vacuoles, cytoplasm and ribosomes fraction of root. The reduction was 72% in cell wall and wall debris fraction and 42% in soluble fraction. A noticeable reduction (68%) was observed in the chloroplast and nuclei fraction of leaf, compared to control plantlets. The mitochondrial fraction of leaf, root and stem had higher accumulation rate of K and the increase was 59, 55 and 474% in leaf, root and stem respectively (Table 15).

The subcellular distribution of Ca in the leaf showed a decreasing trend in all the fractions except fraction IV. There was a prominent reduction in fraction I and II and it was 12 and 29% respectively as compared to the control plantlets. The reduction of Ca in fraction III was lesser as compared to the control plantlets. Higher Ca accumulation was observed in mitochondria fraction of root and stem and it was 184 and 330% respectively over control plantlets (Table 15).

4.11 Ionomics of different plant parts

The concentrations of Na, K, Ca, Mg, Mn, Ni, Mo, Cr, Al, Zn, Fe, and Cu were analysed in order to highlight the impact of NaCl and CuSO₄ on the distribution of elements in various plant components, such as leaves, stems, and roots. The elements under study are divided into three categories: macro, micro, and beneficial elements. The macronutrient category includes elements like K, Mg, and Ca. Micronutrients includes Mn, Fe, Ni, Cu, Zn, and Mo and beneficial elements include Na, Al, and Se.

4.11.1 NaCl treated plantlets

The influence of NaCl (400 mM) treatment on the distribution of various ions were analysed in the *V. inermis* tissues on 20d. In general, the concentration of macronutrients tends to be decreased in all the tissues of *V. inermis*, i.e., leaf, stem and root subjected to NaCl. However, the status of Na

was found to be remarkably increased in the tissues of NaCl treated plants over the control samples, i.e., 75, 559 and 353% increase was observed in the leaf, root and stem tissues respectively. The content of macronutrients namely, K, Mg and Ca sharply declined in the NaCl treated samples as compared to the control samples and the reduction was more prominent in the root tissues. A decrease of 81, 65 and 89% was observed in the root tissues of *V. inermis* with regard to the distribution of K, Mg and Ca respectively. Likewise, 51% reduction was observed in the distribution of K and Ca, and 34% reduction of Mg in the stem tissues. A reduction of 35, 53 and 36% of K, Mg and Ca were observed in the leaf tissues of *V. inermis* subjected to NaCl stress (Table 16).

In the case of micronutrients namely, Mn, Ni, Cu and Zn, the content was significantly declined in all the tissues of *V. inermis* plantlets treated with NaCl. In the case of Mn, 36, 10 and 59% decrease were observed in the leaf, stem and root tissues respectively. Whereas, a sharp and prominent decrease in the Fe content in leaf (238%), and Ni content in root (289%) was observed in plantlets subjected to NaCl. A reduction of 55 and 19% in Fe content was recorded in the root and stem tissues respectively; and 79 and 87% reduction in the Ni content was recorded in the stem and leaf tissues respectively. Likewise, a decrease of 69, 49 and 90% was observed in the Cu content as well as a reduction of 47, 46 and 51% was observed in Zn content of root, stem and leaf tissues respectively. In the case of Mo, the stem tissue had significant accumulation of this elements as compared to control samples, On the other hand, a decline of Mo content was observed in the root and leaf tissues respectively (Table 16).

The distribution of beneficial elements like Cr, Al and Se in *V. inermis* were also influenced by NaCl exposure. Significant reduction was found in the Cr content of NaCl treated plantlets, i.e., a decrease of 7, 74 and 63% was

Table 16. Ionomics of *V. inermis* plantlets (leaves, roots, stem) subjected to 400 mM NaCl on 20d. Values are the means $\pm$ SE of the three different experiments.

Ionome ($\mu\text{g/g DW}$)														
		Na	K	Mg	Ca	Mn	Fe	Ni	Cu	Zn	Mo	Cr	Al	Se
Control	Leaf	36106.79 $\pm$ 1805 ^b	30054.86 $\pm$ 1502.74 ^a	2834.15 $\pm$ 41.70 ^a	199.94 $\pm$ 9.99 ^a	49.01 $\pm$ 2.450 ^a	37.77 $\pm$ 1.88 ^a	0.796 $\pm$ 0.039 ^a	290.01 $\pm$ 14.950 ^a	2.94 $\pm$ 0.147 ^a	2.78 $\pm$ 0.139 ^a	8.86 $\pm$ 0.443 ^a	205.86 $\pm$ 15.293 ^a	1.85 $\pm$ 0.092 ^a
	Root	2647.42 $\pm$ 132.391 ^b	28285.22 $\pm$ 1414.26 ^a	3171.23 $\pm$ 158.56 ^a	154.04 $\pm$ 7.702 ^a	72.17 $\pm$ 3.608 ^a	139.89 $\pm$ 6.99 ^a	0.086 $\pm$ 0.004 ^a	85.47 $\pm$ 4.27 ^a	34.23 $\pm$ 1.711 ^a	6.58 $\pm$ 0.329 ^a	28.01 $\pm$ 1.40 ^a	359.63 $\pm$ 17.981 ^a	0.56 $\pm$ 0.028 ^b
	Stem	1449.55 $\pm$ 72.47 ^b	3266.523 $\pm$ 163.32 ^a	573.33 $\pm$ 28.66 ^a	26.037 $\pm$ 1.301 ^a	4.27 $\pm$ 0.213 ^a	8.37 $\pm$ 0.518 ^a	1.719 $\pm$ 0.085 ^a	5.59 $\pm$ 0.279 ^a	0.699 $\pm$ 0.034 ^a	0.51 $\pm$ 0.025 ^b	4.47 $\pm$ 0.223 ^a	27.78 $\pm$ 1.389 ^a	0.012 $\pm$ 0.0016 ^b
400 mM	Leaf	63055.32 $\pm$ 3152 ^a	18165.85 $\pm$ 908.29 ^b	1836.83 $\pm$ 91.84 ^b	93.56 $\pm$ 4.67 ^b	31.29 $\pm$ 1.56 ^b	60.01 $\pm$ 3.001 ^b	0.591 $\pm$ 0.029 ^b	36.44 $\pm$ 1.822 ^b	0.290 $\pm$ 0.014 ^b	1.37 $\pm$ 0.068 ^b	8.24 $\pm$ 0.412 ^a	240.08 $\pm$ 12.004 ^a	0.080 $\pm$ 0.004 ^b
	Root	17444.39 $\pm$ 872.2 ^a	5297.04 $\pm$ 264.85 ^b	1107.44 $\pm$ 55.37 ^b	17.21 $\pm$ 0.860 ^b	29.33 $\pm$ 1.466 ^b	62.47 $\pm$ 3.123 ^b	0.039 $\pm$ 0.001 ^b	26.07 $\pm$ 1.303 ^b	18.14 $\pm$ 0.907 ^b	2.24 $\pm$ 0.114 ^b	7.17 $\pm$ 0.358 ^b	114.26 $\pm$ 5.713 ^b	1.31 $\pm$ 0.065 ^a
	Stem	6569.62 $\pm$ 328.48 ^a	1609.15 $\pm$ 80.45 ^b	376.53 $\pm$ 18.82 ^b	12.83 $\pm$ 0.641 ^b	3.84 $\pm$ 0.192 ^a	6.81 $\pm$ 0.340 ^a	0.355 $\pm$ 0.017 ^b	2.86 $\pm$ 0.143 ^b	0.374 $\pm$ 0.018 ^b	0.62 $\pm$ 0.031 ^a	1.67 $\pm$ 0.083 ^b	26.53 $\pm$ 1.326 ^a	0.150 $\pm$ 0.007 ^a

Table 17. Ionomics of *V. inermis* plantlets (leaves, roots, stem) subjected to 200 μM CuSO_4 on 20d. Values are the means $\pm$ SE of the three different experiments.

Ionome ($\mu\text{g/g DW}$)														
	Plant parts	Na	K	Mg	Ca	Mn	Fe	Ni	Cu	Zn	Mo	Cr	Al	Se
Control	Leaf	36106.79 $\pm$ 1805 ^b	30054.86 $\pm$ 1502.74 ^a	2834.15 $\pm$ 41.70 ^a	199.94 $\pm$ 9.99 ^a	49.01 $\pm$ 2.450 ^a	37.77 $\pm$ 1.88 ^a	0.796 $\pm$ 0.039 ^a	290.01 $\pm$ 14.950 ^a	2.94 $\pm$ 0.147 ^a	2.78 $\pm$ 0.139 ^a	8.86 $\pm$ 0.443 ^a	205.86 $\pm$ 15.293 ^a	1.85 $\pm$ 0.092 ^a
	Root	2647.42 $\pm$ 132.391 ^b	28285.22 $\pm$ 1414.26 ^a	3171.23 $\pm$ 158.56 ^a	154.04 $\pm$ 7.702 ^a	72.17 $\pm$ 3.608 ^a	139.89 $\pm$ 6.99 ^a	0.086 $\pm$ 0.004 ^a	85.47 $\pm$ 4.27 ^a	34.23 $\pm$ 1.711 ^a	6.58 $\pm$ 0.329 ^a	28.01 $\pm$ 1.40 ^a	359.63 $\pm$ 17.981 ^a	0.56 $\pm$ 0.028 ^b
	Stem	1449.55 $\pm$ 72.47 ^b	3266.523 $\pm$ 163.32 ^a	573.33 $\pm$ 28.66 ^a	26.037 $\pm$ 1.301 ^a	4.27 $\pm$ 0.213 ^a	8.37 $\pm$ 0.518 ^a	1.719 $\pm$ 0.085 ^a	5.59 $\pm$ 0.279 ^a	0.699 $\pm$ 0.034 ^a	0.51 $\pm$ 0.025 ^b	4.47 $\pm$ 0.223 ^a	27.78 $\pm$ 1.389 ^a	0.012 $\pm$ 0.0016 ^b
200 mM	Leaf	19988.3 $\pm$ 999.415	4717.66 $\pm$ 235.883	3980 $\pm$ 199	100.35 $\pm$ 5.017	72.06 $\pm$ 3.603	40.51 $\pm$ 2.025	3.58 $\pm$ 0.179	26.15 $\pm$ 1.307	2.95 $\pm$ 0.147	1.3 $\pm$ 0.065	6.84 $\pm$ 0.342	349.76 $\pm$ 17.488	1.05 $\pm$ 0.052
	Root	2693.73 $\pm$ 134.686	10537.83 $\pm$ 526.8915	1446.43 $\pm$ 72.321	61.32 $\pm$ 3.066	15.20 $\pm$ 0.76	74.01 $\pm$ 3.70	11.99 $\pm$ 0.599	5144.04 $\pm$ 257.202	7.23 $\pm$ 0.361	10.39 $\pm$ 0.519	22.54 $\pm$ 1.127	354.57 $\pm$ 17.728	2.09 $\pm$ 0.104
	Stem	1556.32 $\pm$ 77.816	4288.81 $\pm$ 214.4405	798.74 $\pm$ 39.937	27.13 $\pm$ 1.356	5.17 $\pm$ 0.258	20.97 $\pm$ 1.048	2.34 $\pm$ 0.117	469.55 $\pm$ 23.477	1.41 $\pm$ 0.0705	0.698 $\pm$ 0.0349	4.77 $\pm$ 0.238	57.23 $\pm$ 2.861	0.77 $\pm$ 0.038

observed in the leaf, root and stem tissues respectively. Similarly, the distribution of Al was found to be decreased, in the root and stem tissues of *V. inermis* subjected to NaCl as compared to the control samples. On the other hand, in the leaf tissue significant increase in the Al content was observed as compared to the control plantlets (17%). The distribution of Se was found to be remarkably increased in the stem of *V. inermis*, i.e., 1059% increase over the control samples. In the root tissue also Se was increased significantly (133%) over the control roots. However, in the leaf tissue Se content decreased significantly (96%) compared to the control leaves (Table 16).

4.11.2 CuSO₄ treated plantlets

The influence of CuSO₄ (200 µM) treatment on the distribution of various ions were analysed in the *V. inermis* tissues. The concentration of macronutrients (K, Mg, Ca) tends to have drastic variations in all the tissues of *V. inermis*, i.e., leaf, stem and root subjected to CuSO₄. Among the different plant parts studied, roots exhibit a reduction in K content (32%) and other two macronutrients such as Mg and Ca recorded an increase. High level of increase in Ca (153%) was observed in root tissue in response to CuSO₄. All of the macronutrients that were examined in the case of stem tend to drop (30-33%) in response to 200 µM CuSO₄. The leaf macronutrient level was observed to be increased in the case of elements such as K and Ca but Mg content was decreased (38%) (Table 17).

The micronutrients (Mn, Fe, Ni, Cu, Zn, and Mo) profile of the roots recorded an increasing pattern except in the case of Zn, where there was a decline of about 17%. In response to 200 µM CuSO₄, the amount of other elements, including Mn, Fe, Ni, Cu, and Mo, increased. The maximum peak of increase was observed in the case of Cu, followed by Mo, Ni, Mn, and Fe (Cu>Mo>Ni>Mn>Fe). In the case of stem, all the micronutrients under study

were declined except Cu, which increased by 66-fold over control plantlets. Exposure of plantlets to 200 μM CuSO_4 , altered the absorption rate of elements such as Ni, Fe, Zn, Mn and Mo. These elements decreased drastically and the reduction was higher in the case of Ni followed by Fe, Zn, Mn and Mo (Table 17).

The status of the beneficial elements such as Al and Se was found to be remarkably increased in the root and leaf tissues of CuSO_4 treated plantlets over the control samples. The increase was higher in roots and was 125 and 129% in the case of Al and Se respectively over control plantlets. The increase of Al and Se in the leaves of CuSO_4 treated plantlets was comparatively less than in the case of roots. The Na content in the different plant parts after exposure to CuSO_4 drastically declined as compared to control plantlets. The higher reduction in Na content was observed in roots (40%), followed by stem (29%) and leaf (10%) (Table 17).

4.12 C, H and N analysis

4.12.1 Leaves

The distribution of major macro elements such as C, H and N were analysed in the leaves and root samples to analyse the impact of NaCl and CuSO_4 on *V. inermis* on 20d. The exposure of NaCl and CuSO_4 drastically changed the distribution pattern of C, H and N. The leaves C content in the plants subjected to NaCl was reduced in different treatments. The leaves of the control plantlets have $47.18 \pm 2.35\%$ C content and was reduced to $35.51 \pm 1.77\%$ in the plantlets treated with 400 mM NaCl. Similar kind of reduction was also observed in H content. The higher NaCl treatment (400 mM) interferes with the accumulation of H content also. While the H content was $6.05 \pm 0.302\%$ in control, in NaCl treated plantlets, it was only $4.77 \pm 0.23\%$. The N content in the leaves show a decreasing trend except in

Table 18. C, H and N analysis in the leaf and root of *V. inermis* plantlets subjected to varying concentrations of NaCl (0, 100, 200, 300 and 400 mM) and CuSO₄ (0, 100 and 200 µM). Values are the mean ±SE of three different experiments. Different alphabetical letters indicate significant difference between treatments (Duncan's test $p \leq 0.05$).

NaCl (mM) Treatment	Leaf					Root				
	C (%)	H (%)	N (%)	C/N ratio	C/H ratio	C (%)	H (%)	N (%)	C/N ratio	C/H ratio
0	47.18±2.35 ^a	6.05±0.302 ^a	3.81±0.190 ^b	12.38±0.619	7.79±0.389	36.91±1.84 ^b	5.01±0.251 ^a	3.38±0.169 ^b	10.92±0.546	7.36±0.368
100	42.61±2.13 ^b	5.69±0.284 ^b	4.41±0.220 ^a	9.68±0.484	7.48±0.374	39.45±1.97 ^a	5.04±0.248 ^a	3.47±0.187 ^a	11.36±0.568	7.82±0.391
200	40.64±2.03 ^c	5.09±0.254 ^c	3.01±0.150 ^c	13.50±0.675	7.98±0.399	35.94±1.79 ^c	4.74±0.237 ^b	2.65±0.132 ^c	13.56±0.678	7.58±0.371
300	32.91±1.64 ^e	4.42±0.221 ^e	1.15±0.057 ^d	28.60±1.43	7.44±0.373	35.52±1.77 ^c	4.79±0.239 ^b	2.05±0.102 ^d	17.32±0.866	7.41±0.372
400	35.51±1.77 ^d	4.77±0.238 ^d	0.967±0.048 ^e	36.72±1.83	7.44±0.371	35.76±1.788 ^c	4.83±0.241 ^b	1.33±0.066 ^e	26.88±1.344	7.40±0.318
CuSO ₄ (µM) Treatment	Leaf					Root				
	C (%)	H (%)	N (%)	C/N ratio	C/H ratio	C (%)	H (%)	N (%)	C/N ratio	C/H ratio
0	47.18±2.35 ^a	6.05±0.302 ^a	3.81±0.190 ^b	12.38±0.619	7.79±0.389	36.91±1.99 ^c	5.01±0.252 ^b	3.38±0.169 ^a	10.92±0.546	7.36±0.368
100	41.19±2.05 ^b	2.41±0.120 ^c	5.73±0.286 ^a	7.18±0.359	7.61±0.380	42.69±2.13 ^a	5.58±0.279 ^a	2.28±0.114 ^b	18.72±0.936	7.65±0.382
200	38.39±1.91 ^c	5.32±0.266 ^b	2.675±0.133 ^c	14.35±0.717	7.21±0.360	39.11±1.95 ^b	5.53±0.276 ^a	1.98±0.099 ^c	19.75±0.987	7.07±0.353

the plantlets treated with 100 mM NaCl. The N content of control leaves was $3.81 \pm 0.190\%$, while it was only $0.967 \pm 0.048\%$ in leaves of plantlets exposed to 400 mM NaCl. The plantlets treated with NaCl (100 mM) showed a mild increase (Table 18).

The plantlets exposed to CuSO_4 also showed variation in the accumulation of C, H and N of the leaves. The C and H content was reduced than control plantlets on exposure to CuSO_4 and the reduction was progressive with increasing concentration of CuSO_4 in the medium. The leaves C content was $41.19 \pm 2.05\%$ and $38.39 \pm 1.91\%$ in plantlets exposed to 100 and 200 μM CuSO_4 respectively. Similar to this, H content was also reduced in response to 100 and 200 μM CuSO_4 . In contrast to this, a gradual increase was found in N content of the plantlets exposed to 100 μM CuSO_4 and it was $5.73 \pm 0.286\%$. But there was a reduction in N content of the plantlets exposed to 200 μM CuSO_4 and the value was $2.67 \pm 0.133\%$ (Table 18).

4.12.2 Roots

The pattern of macro elements (C, H and N) distribution was almost similar to that of leaves. The C, H and N content in roots of the plantlets exposed to NaCl (200 mM and above) was reduced. The C content in roots of plantlets exposed to 400 mM NaCl was $35.76 \pm 1.78\%$, while in control plantlets it was $36.91 \pm 1.84\%$. In the case of H, the trend was similar as that of C. The plantlets exposed to 400 mM NaCl had only $4.83 \pm 0.241\%$ of H, while it was $5.01 \pm 0.250\%$ in the control. The N content showed a reduction trend except the roots of the plantlets exposed to 100 mM NaCl, where there was a slight increase (3.47 ± 0.173) than the roots of the control plantlets (3.38 ± 0.169). The N content of the roots exposed to 400 mM NaCl was only $1.33 \pm 0.066\%$ (Table 18).

The plantlets exposed to CuSO_4 showed a similar trend of reduction in C, H and N content. The reduction was more in the case of C and N content and was negligible in the case of H content. The C content of the roots exposed to 200 μM CuSO_4 was only $39.11 \pm 1.95\%$ while in the roots of control plantlets it was $42.69 \pm 2.13\%$. The N content was 2.28 ± 0.114 and 1.98 ± 0.097 in plantlets exposed to 100 and 200 μM CuSO_4 respectively compared with the roots of control plantlets (3.38 ± 0.169) (Table 18).

4.12.3 Carbon to nitrogen and carbon to hydrogen ratio (C/N & C/H ratio)

4.12.3.1 Leaves

The ratio of carbon to nitrogen and carbon to hydrogen was varied in both leaves and roots with respect to the treatment. The C/N ratio of the leaves treated with NaCl showed an increasing trend with respect to increasing NaCl concentrations except in 100 mM NaCl. The maximum C/N ratio was found in the leaves of plantlets exposed to 300 and 400 mM NaCl and the increase was 131 and 197% respectively than control plantlets. The increase in the C/N ratio of the plantlets exposed to 200 mM NaCl was lesser and the increase was only 9% than control plantlets. In the case of plantlets exposed to 200 μM CuSO_4 , C/N ratio increased by 16% over control plantlets. In contrast to this, exposure to lower concentration of CuSO_4 (100 μM) caused decline in the C/N ratio (42%) compared with control plantlets (Table 18).

The carbon to hydrogen ratio (C/H) was essentially the same across all treatments (both NaCl and CuSO_4). In the case of NaCl treatment, the C/H ratio was similar in the leaves of control and plantlets exposed to 200 mM NaCl. Similar way, the mild concentration of CuSO_4 (100 μM) showed similar value as that of control (Table 18).

4.12.3.2 Roots

C/N ratio in the roots of NaCl treated plantlets enhanced with increasing concentration of NaCl. A sharp increase was found in the plantlets exposed to 300 and 400 mM NaCl and the enhancement was 57 and 146% respectively than the control plantlets. The increase in C/N ratio of the plantlets exposed to 100 mM NaCl was insignificant compared with control plantlets. The exposure of *V. inermis* plantlets to CuSO₄ enhanced the C/N ratio and the enhancement was maximum in the plantlets treated with 200 μ M CuSO₄. The percentage of increase was found to be 71 and 81% in plantlets subjected to 100 and 200 μ M CuSO₄ respectively over the control plantlets. The root carbon to hydrogen ratio (C/H) was essentially the same across all treatments (both NaCl and CuSO₄) (Table 18).

4.13 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy analysis was carried out to analyse the changes in functional groups associated with various carbohydrate structures, phenols, or aldehydes of leaves and roots in response to NaCl and CuSO₄ exposure in *V. inermis*. The IR spectra displays different absorption peaks at various wave numbers, which are present in the leaves of control, NaCl and CuSO₄ treated plantlets.

4.13.1 NaCl treated plantlets

The leaves of the NaCl treated plantlets exhibited a strong and broad absorption peak at 3421 cm⁻¹ representing the -OH stretching vibration equivalent to that of alcohol. It represents the -OH functional group of polyphenols, carbohydrates and proteins. A medium sized absorption peak at 2929 cm⁻¹ represents the C-H stretching of alkene group and aliphatic stretching group. The absorption peak at 2352 cm⁻¹ represents C=C and it possibly indicates C=C of lignin. It was not found in the plantlets treated

with 100 mM NaCl. The strong absorption peak at 1625 cm^{-1} represents the C=O amide I band. A medium size peak at 1424 cm^{-1} indicates the stretching vibrations of -C=O inorganic carbonate (Fig. 28).

The strong and broad absorption peak at 3427 cm^{-1} represents the -OH functional group of alcohols, carbohydrates, proteins and polyphenols. This specific band was widened and shallow in the roots of NaCl treated plantlets. The peak at 2922 cm^{-1} represents C-H stretching of alkene group and aliphatic stretching group. This peak show characteristic difference in the roots of control and NaCl treated plantlets. This was more refined and sharper peak was found in 300 and 400 mM NaCl treated plantlets. The strong band at 1643 cm^{-1} represents C=C of stretching in alkene. The intensity of this peak was varied among treatments and it was sharper in plantlets treated with 300 mM NaCl. A peak at 1384 cm^{-1} represents C-H bending of aldehyde or alkane. A strong and broad peak at 1049 cm^{-1} was found in all the samples but it was sharper and refined in the roots of 300 mM NaCl treated plantlet and it represents the CO-O-CO stretching of anhydride functional groups (Fig. 28).

4.13.2 CuSO₄ treated plantlets

The leaves of the CuSO₄ treated plantlets exhibited a strong and broad absorption peak at 3395 cm^{-1} representing the -OH stretching vibration equivalent to that of alcohol. It also represents the -OH functional group of polyphenols, carbohydrates and proteins. A medium sized absorption peak at 2929 cm^{-1} represents the C-H stretching of alkene group and aliphatic stretching group. The strong absorption peak at 1636 cm^{-1} represents the -O stretch (amides), N-H bend (nitro compounds, amines), C=O stretch (carboxylic acids, ketones), C=C (alkenes, benzenes) (proline), C=O amide I band. A medium size peak at 1410 cm^{-1} indicates the stretching vibrations of -C=O inorganic carbonate. The peak at 1248 cm^{-1} corresponds

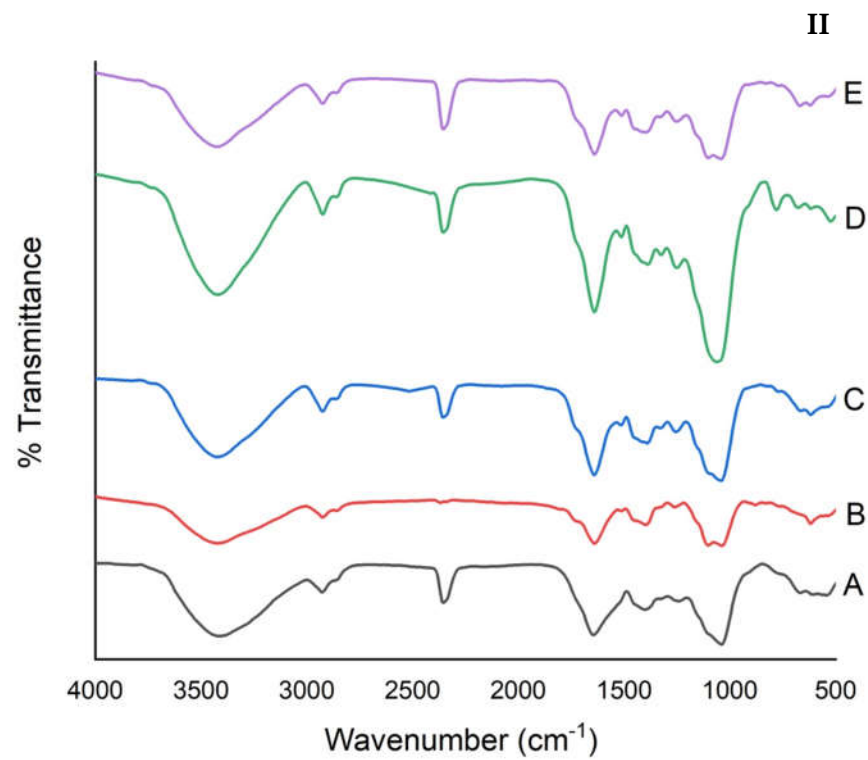
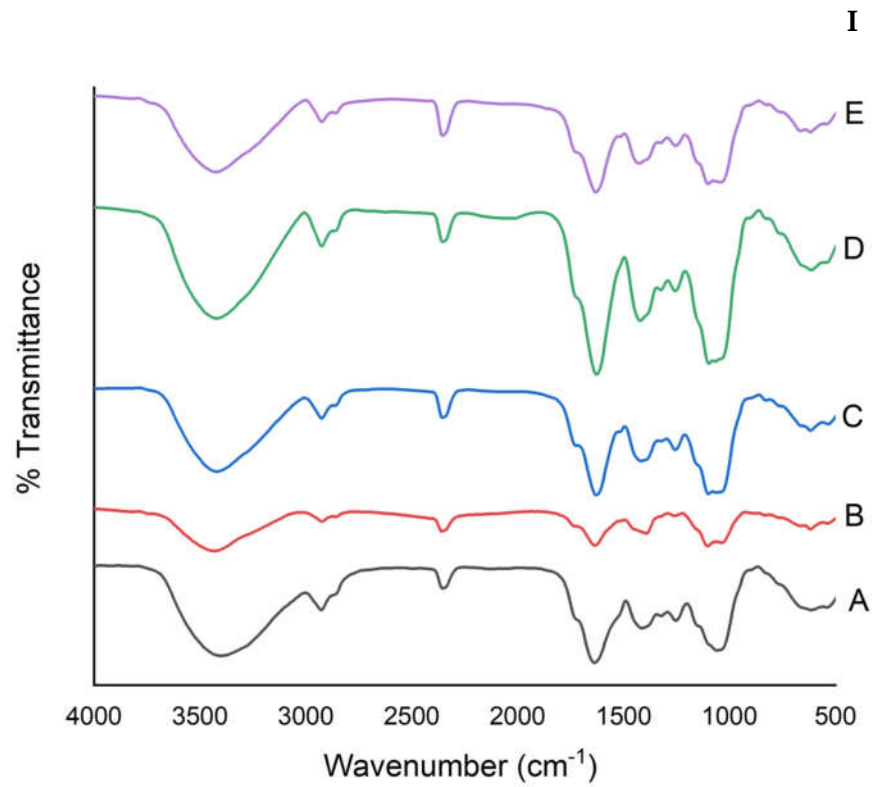


Figure 28. FTIR spectra of the leaf (I) and root (II) of *V. inermis* plantlets subjected to varying concentrations of NaCl (A-0, B-100, C-200, D-300 and E-400 mM) on 20d.

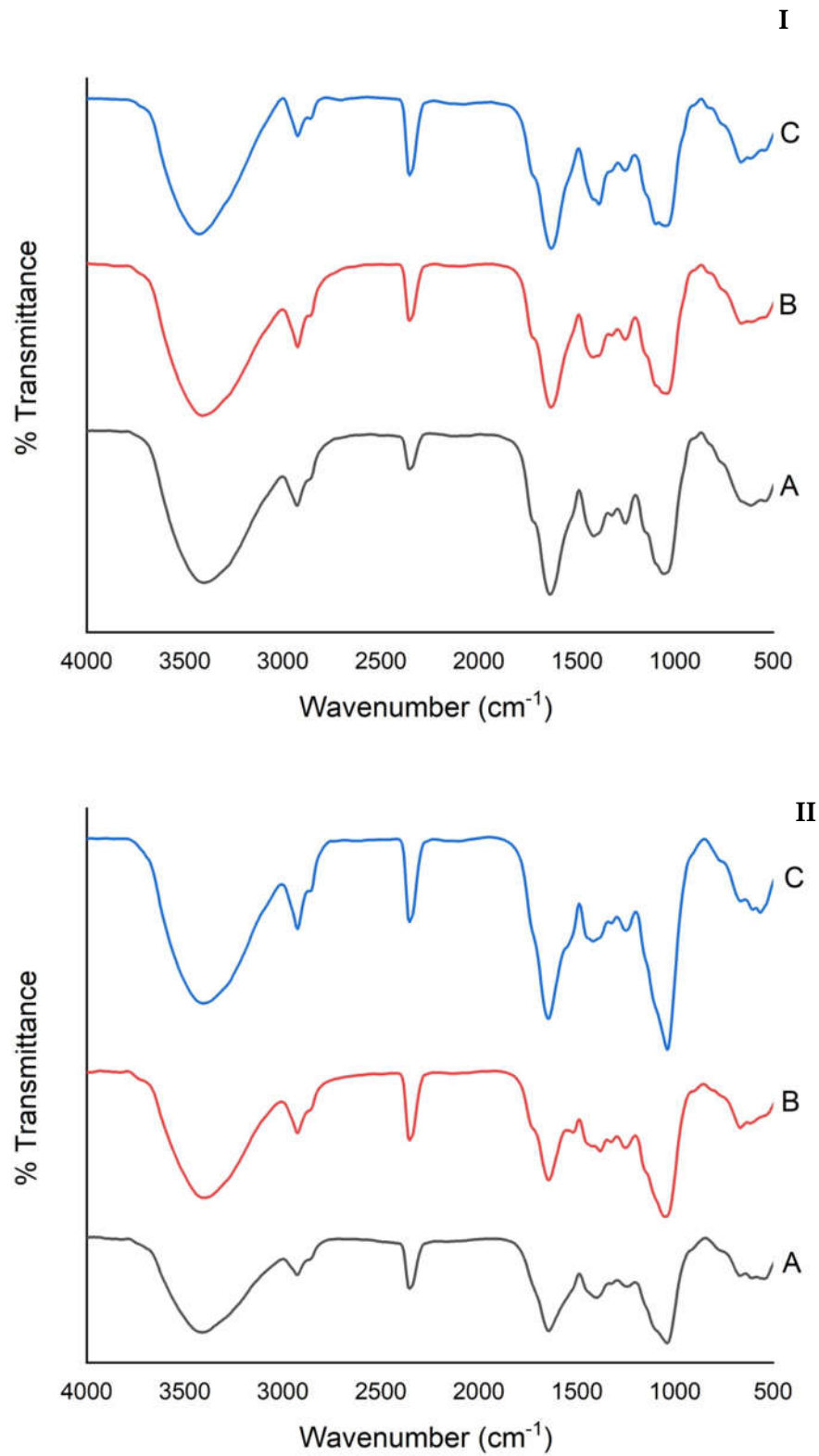


Figure 29. FTIR spectra of the leaf (I) and root (II) of *V. inermis* plantlets subjected to varying concentrations of CuSO_4 (A-0, B-100 and C-200 μM) on 20d.

to the C-N stretch (amines), C-N amide III band, C-O stretch (esters), C-O stretch (Ethers, Alcohols), O-H band (carboxylic acids). The strong peak at 1055 cm^{-1} represents the S=O stretching (sulfoxides), C-N stretch (amines), C-O stretch (esters, ether, alcohol), =C-H bend (alkenes) (rhamnogalacturonan). A weak absorption peak at 667 cm^{-1} varied in intensity from control to CuSO_4 treatment and it indicates C-N stretch (amines), =C-H bend (benzene), C-C stretch (chlorides) (Fig. 29).

The strong and broad absorption peak at 3412 cm^{-1} represents the -OH functional group of alcohols, carbohydrates, proteins and polyphenols. This specific band is more widened and shallower in control and it is more sharp and less widened in the roots of CuSO_4 treated plantlets. The peak at 2928 cm^{-1} represents C-H stretching of alkene group and aliphatic stretching group. This peak show characteristic difference in the roots of control and CuSO_4 treated plantlets. More refined and sharp peak was found in $200\text{ }\mu\text{M}$ CuSO_4 treated plantlets but it becomes shallow in others. The strong band at 1648 cm^{-1} indicates C-O stretch (amides), N-H bend (nitro compounds, amines), C=O stretch (carboxylic acids, ketones), C=C (alkenes, benzenes) (proline), C=O amide I band and C=O stretching of alkenes. This peak was narrow and sharp in treatments with higher CuSO_4 concentration and more widened and shallower in others. This indicates the interactions of this specific functional group with other molecules. A weak and specific band was observed at 1397 cm^{-1} and denotes CH and CH_2 aliphatic bending group. This peak shifted in response to CuSO_4 treatment and the peak was at 1376 cm^{-1} representing the same functional group. A characteristic peak was observed in 1255 cm^{-1} representing C-N amide III band and it was absent or negligible in control and was distinct in CuSO_4 treated plantlets. A strong and broad peak at 1042 cm^{-1} was found in all the samples but it was sharp and refined in the roots of $200\text{ }\mu\text{M}$ CuSO_4 treated plantlet and it represents the CO-O-CO stretching of anhydride functional groups (Fig. 29).

4.14 Anatomical studies

4.14.1 Leaf

The cross section of the leaf lamina of *V. inermis* on 20d of growth shows a waxy cuticle with single layered epidermis on both adaxial and abaxial sides. The abaxial epidermal cells are cylindrical, but the adaxial epidermal cells are nearly rectangular. The stomata are absent or very less in adaxial side and are present abundantly on the abaxial side (hypostomatic). The stomata are anomocytic. Presence of glandular trichomes were noticed on both upper and lower epidermis (Fig. 31). The mesophyll cells are made up of 1-2 layers of palisade cells and 9-10 layers of spongy parenchyma tissues. The vascular bundles are closed, collateral and with endarch xylem. In the vascular bundle, the xylem is located towards the adaxial surface and the phloem is situated towards the abaxial surface. A sheath of parenchymatous cells known as the bundle sheath encircles the vascular bundles (Fig. 30).

The leaves of the plantlets exposed to 400 mM NaCl showed no characteristic changes or damages in the anatomy. An increased number of trichomes are observed in response to NaCl stress as compare with control. A comparable change in the xylary elements in the vascular cylinder were noted in the leaves of treated plantlets compared with control plantlets. The staining test with phloroglucinol showed the presence of highly lignified cells as cherry pink or fuchsia colour in the leaf vascular region of plantlets subjected to 400 mM NaCl compared to control plantlets (Fig. 30). Similar observations were also noticed in the leaves of the plantlets exposed to 200 μ M CuSO₄. The staining with toluidine blue O in the CuSO₄ treated leaves showed bright blue coloured regions specifically in the vascular cylinder. This was clearly evident from the figures and was identified as xylary elements. The bright blue colour indicates the deposits of lignin and tannins

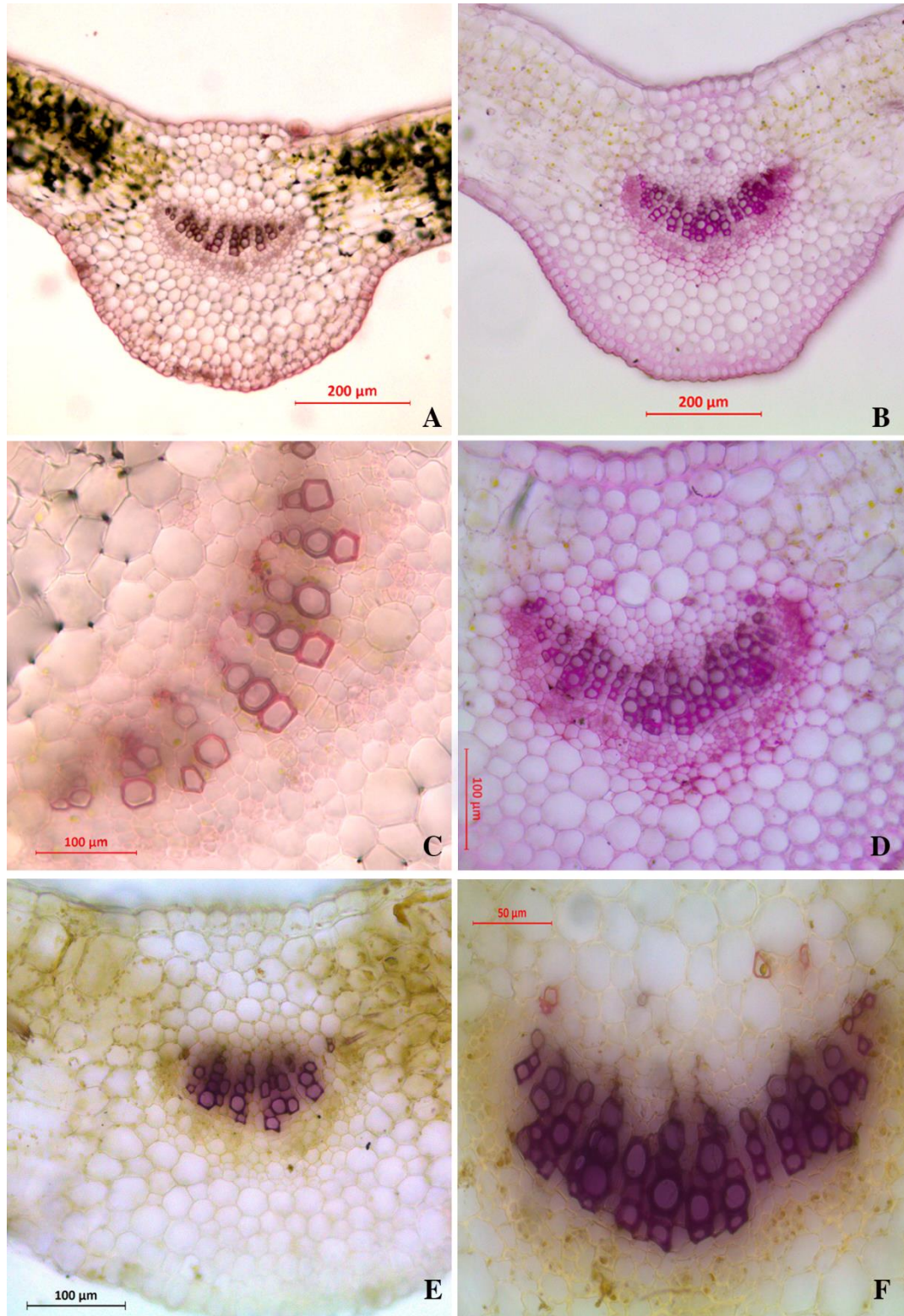


Figure 30. Anatomical variations and Phloroglucinol-HCl test for lignin in the leaves of *V. inermis* plantlets (cross-section view and vascular region) subjected to 400 mM NaCl. A- Control cross-section view, B- 400 mM NaCl cross-section view, C-control vascular region, D-400 mM NaCl vascular region, E-Control lignified cells, F-400 mM NaCl lignified cells.

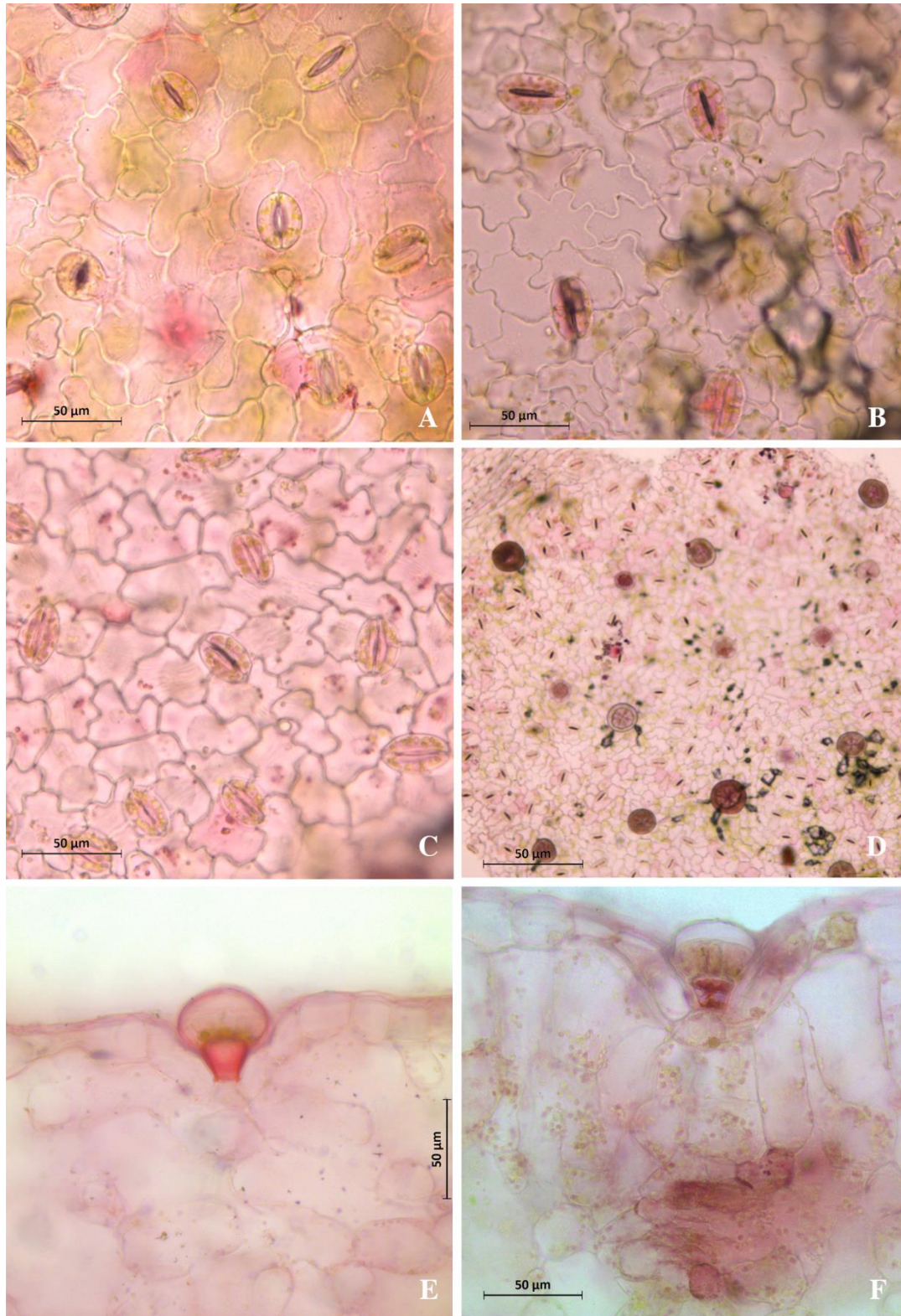


Figure 31. Anomocytic stomata and glandular trichomes of *V. inermis* leaves subjected to 400 mM NaCl and 200 µM CuSO₄. A- Control lower surface view, B- 400 mM NaCl lower surface view, C- 200 µM CuSO₄ lower surface view, D- Glandular trichomes and stomata surface view, E and F- Glandular trichomes with multicellular head.

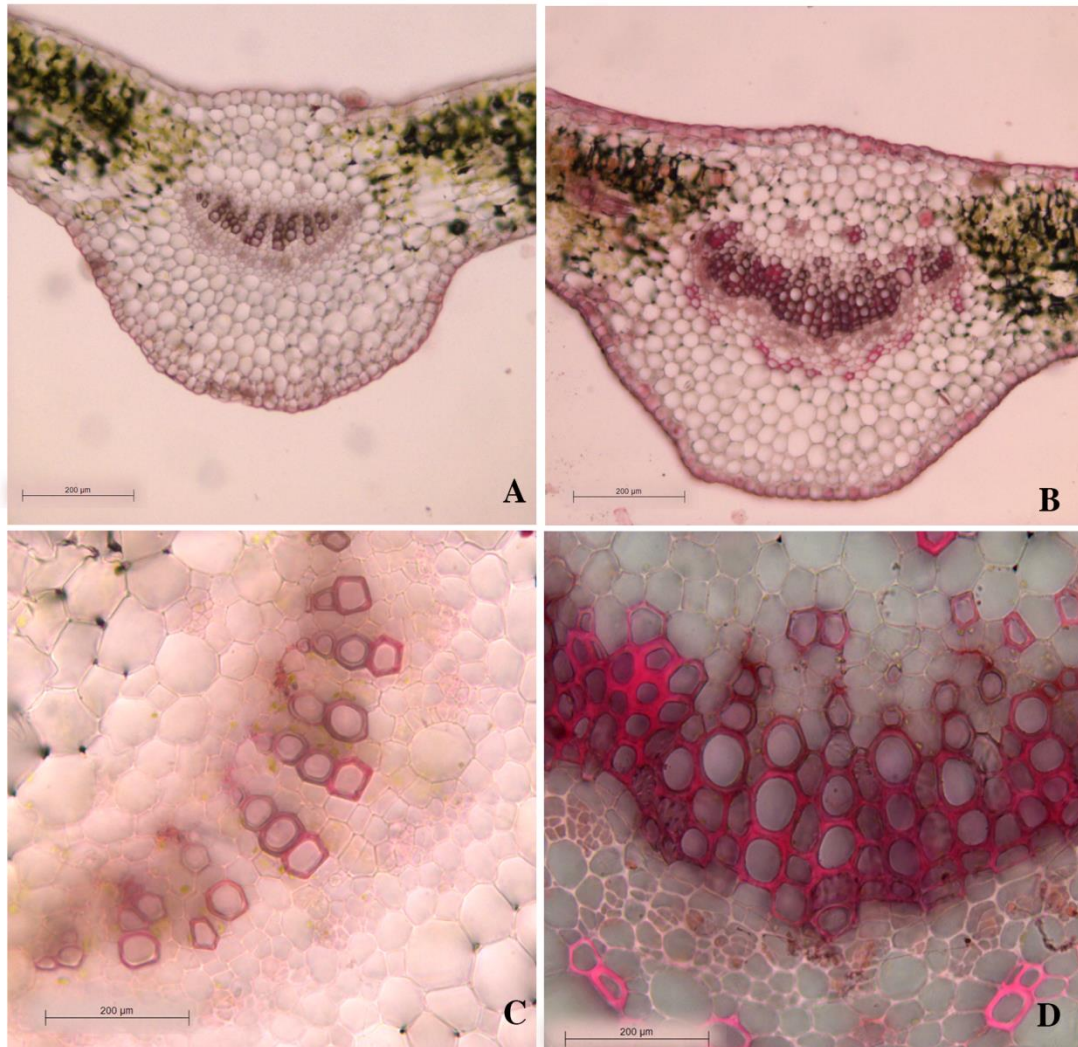


Figure 32. Anatomical variations in the leaves of *V. inermis* plantlets (cross-section view and vascular region) subjected to 200 μM CuSO_4 . A- Control cross-section view, B- 200 μM CuSO_4 cross-section view, C - Control vascular region, D- 200 μM CuSO_4 vascular region.

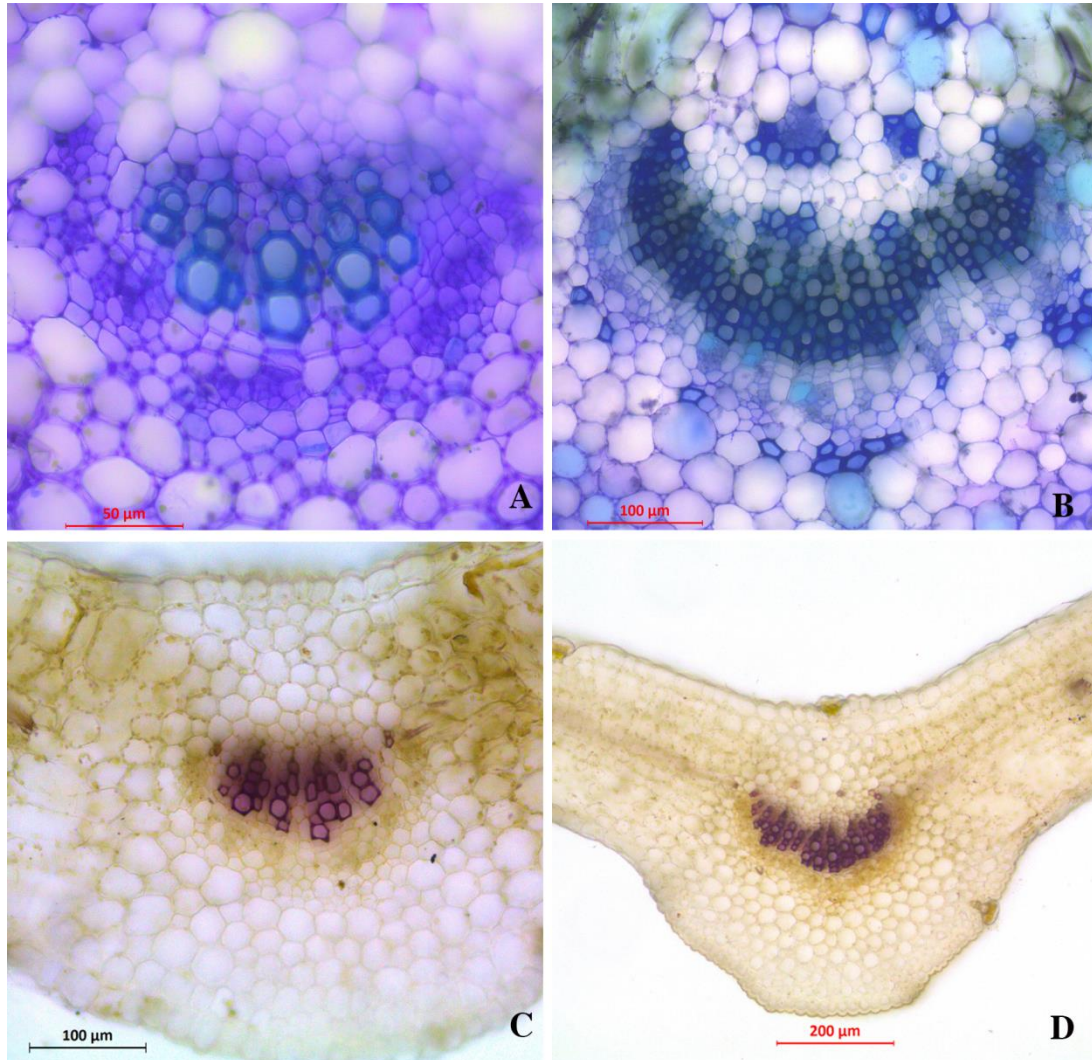


Figure 33. Toluidine staining (A and B) and phloroglucinol-HCl test for lignin (C and D) in the leaves of *V. inermis* plantlets (cross-section view and vascular region) subjected to 200 μM CuSO₄. A- Control vascular region, B- 200 μM CuSO₄ vascular region, C- Control lignified cells, D- 400 mM NaCl lignified cells.

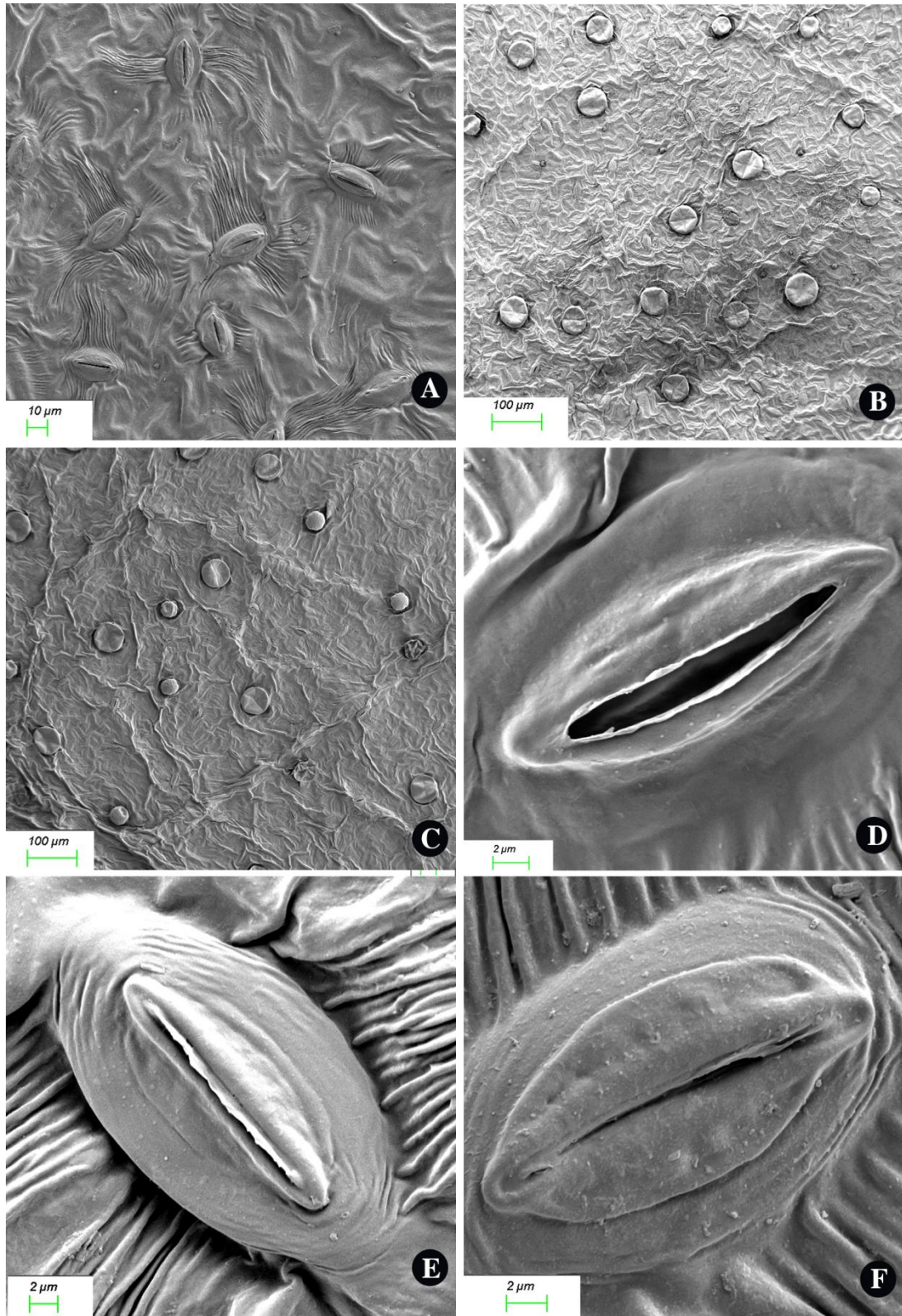


Figure 34. Scanning electron micrograph of *V. inermis* leaves (lower surface view) subjected to 400 mM NaCl and 200 μM CuSO₄. A- Lower surface view control, B- Lower surface view 400 mM NaCl, C- Lower surface view 200 μM CuSO₄, D- Stoma control, E- Stoma 400 mM NaCl, F- Stoma 200 μM CuSO₄.

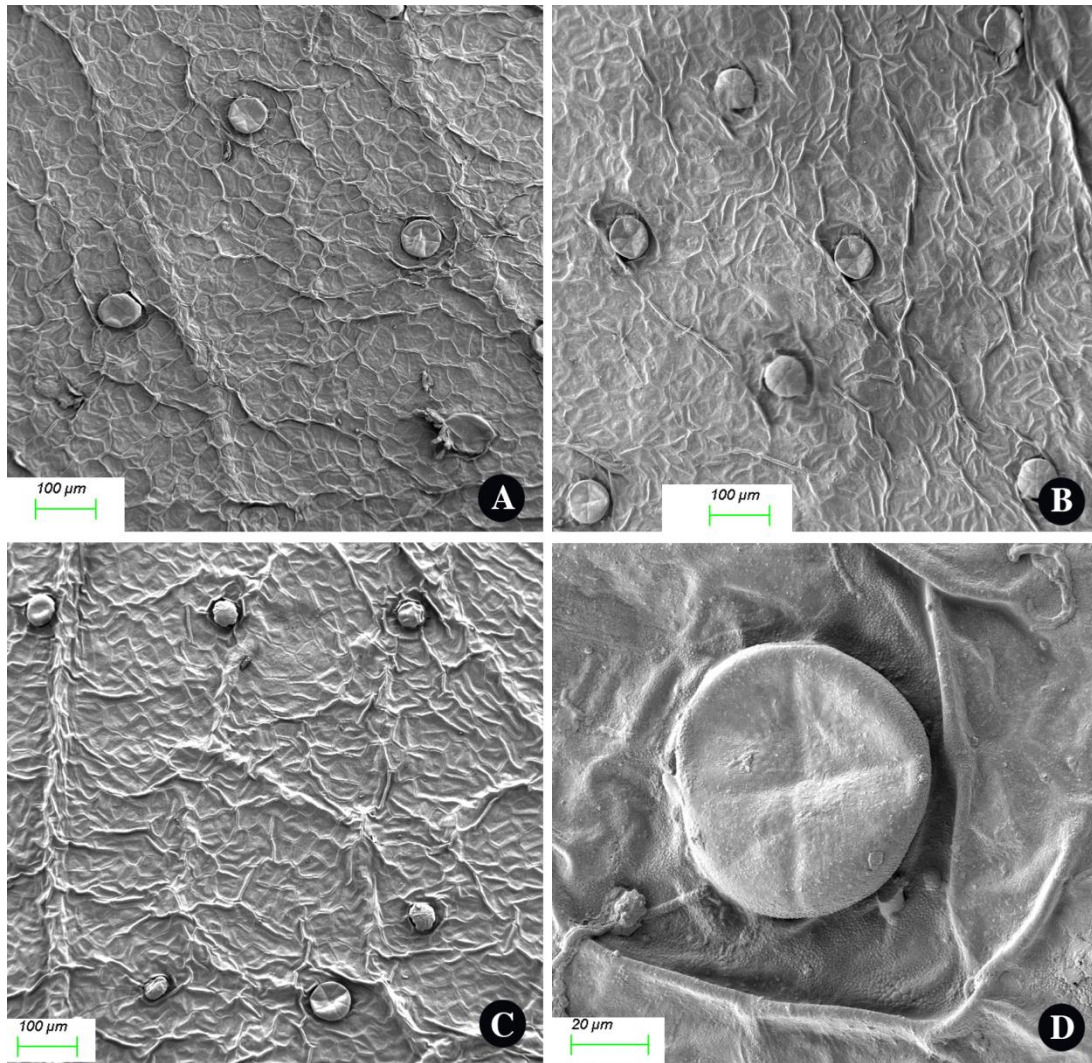


Figure 35. Scanning electron micrograph of *V. inermis* leaves (upper surface view) subjected to 400 mM NaCl and 200 μM CuSO₄. A- Upper surface view control, B- Upper surface view 400 mM NaCl, C- Upper surface view 200 μM CuSO₄, D- Surface view of single enlarged glandular trichome.

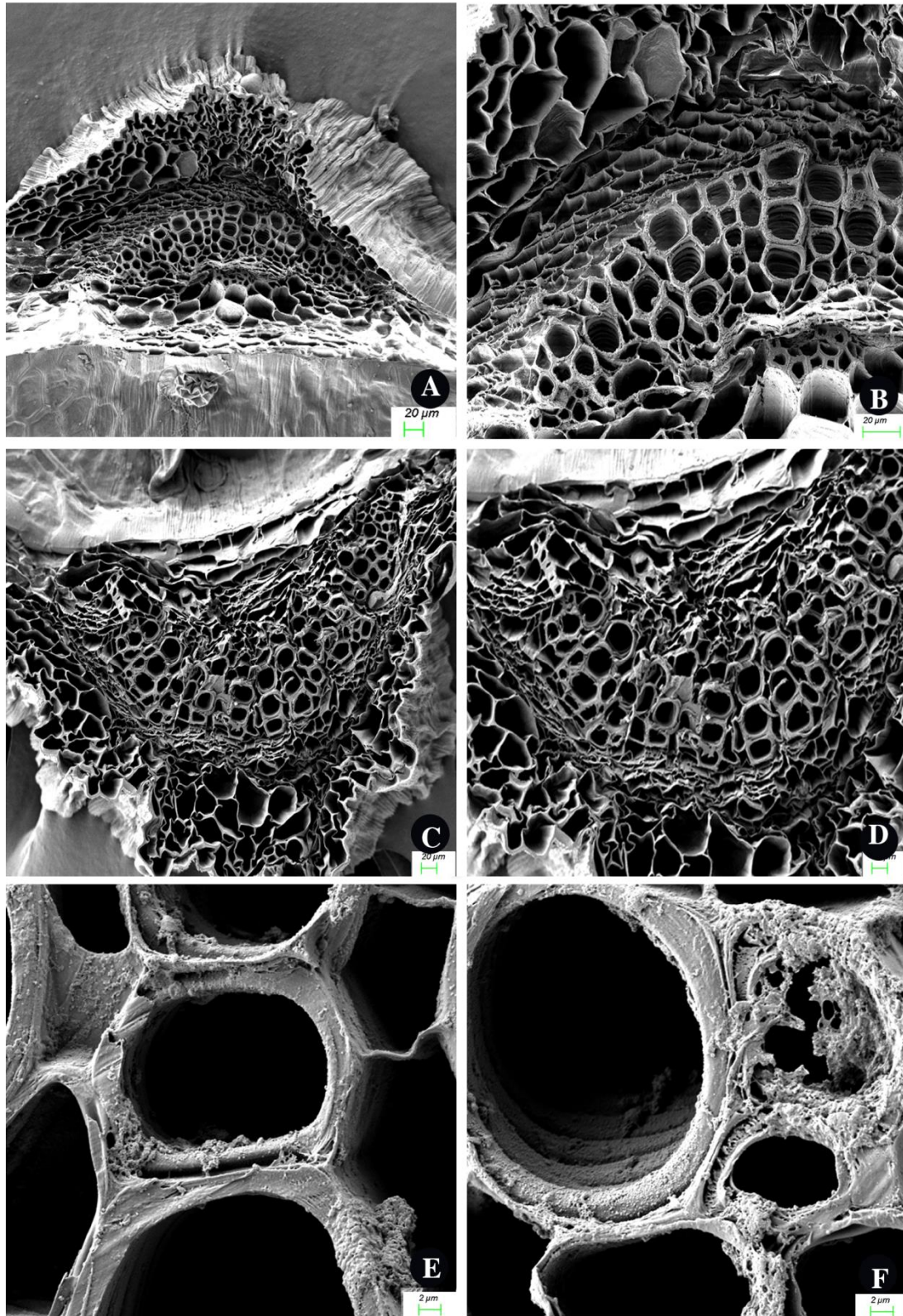


Figure 36. Scanning electron micrograph of *V. inermis* leaves subjected to 400 mM NaCl. A- Control cross-section view, B- 400 mM NaCl cross-section view, C- Control vascular region, D- 400 mM NaCl vascular region, E- Control xylem wall, F- 400 mM NaCl xylem wall.

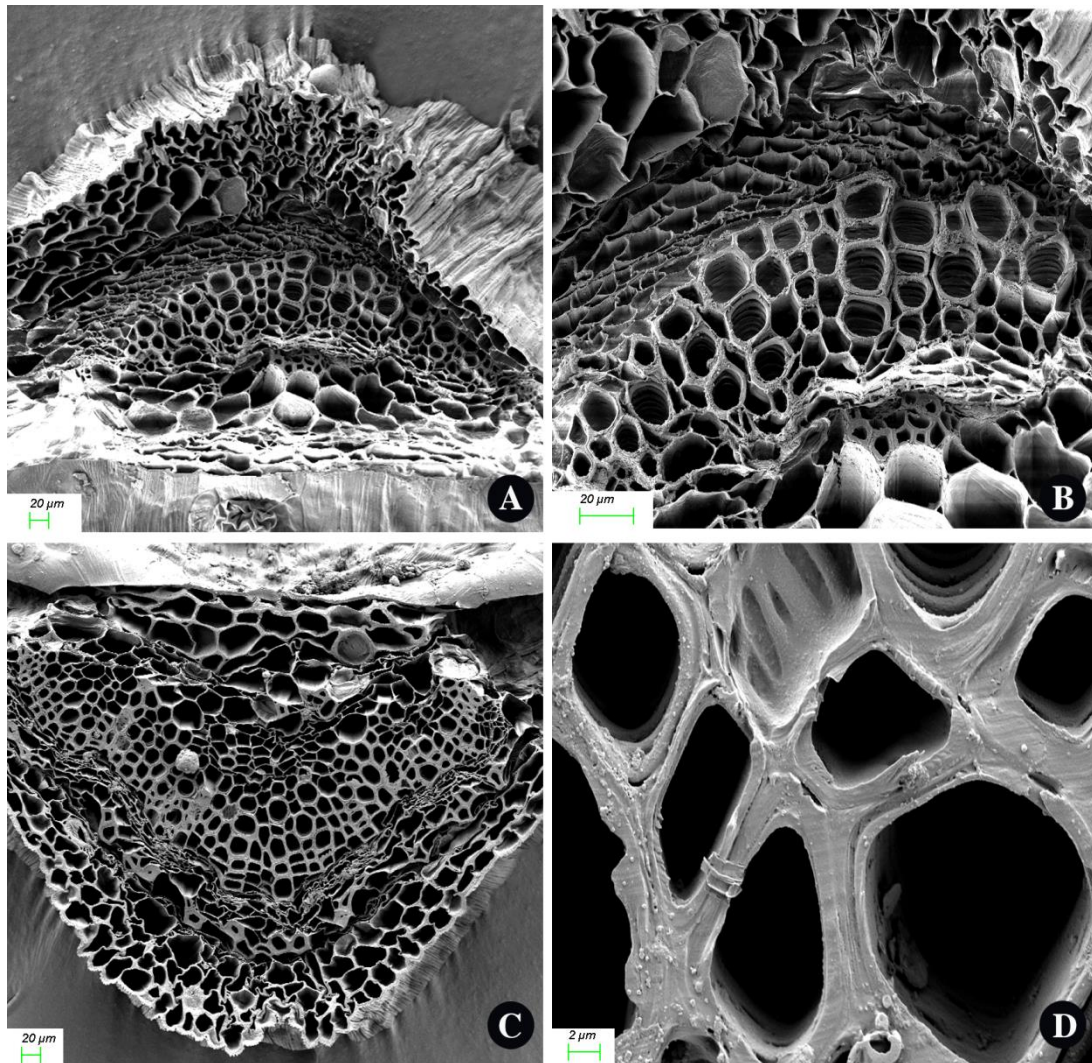


Figure 37. Scanning electron micrograph of *V. inermis* leaves subjected to 200 μM CuSO₄. A- Control cross-section view, B- Control vascular region, C- 200 μM CuSO₄ cross-section view, D- 200 μM CuSO₄ xylem wall.

in the cells (Fig. 32 and 33). The phloroglucinol staining in the CuSO₄ treated leaves showed specific lignin depositions and it was higher in CuSO₄ treated leaves as compared with control leaves (Fig. 33). The shape of the xylem vessels was altered in both NaCl and CuSO₄ treatments and the shape of the vessels in control leaves is almost polygonal; and it got transformed to almost round in shape on exposure to NaCl and CuSO₄.

The scanning electron microscopic studies in the leaves of *V. inermis* on 20d of 400 mM NaCl exposure showed the presence of anomocytic stomata scattered throughout the abaxial surface of the lamina. The stomata were completely open in the lamina of the control plantlets but partially or fully closed in 400 mM NaCl treated plantlets (Fig. 34). Similar observation was also noted in the CuSO₄ treated leaves. The number of trichomes was increased in response to 400 mM NaCl and also in the leaves of CuSO₄ treated plantlets compared with control plantlets where it was too less (Fig. 35). The wall thickness of the xylem vessels in leaves was increased in both the treatments and the increase was 120% in 400 mM NaCl treated plantlets and was 331% in CuSO₄ treated plantlets as compared with the control (Fig. 36 and 37).

Table 19. Xylem wall thickness of leaves and root of *V. inermis* subjected to 400 mM NaCl and 200 μ M CuSO₄. Values are the means $\pm$ SE of the three different experiments.

Plant Sample	Thickness of xylem wall (μ m)	
	Control	400 mM NaCl
Leaf	1.20 $\pm$ 0.091 ^b	2.66 $\pm$ 0.20001 ^a
Root	2.168 $\pm$ 0.190 ^b	4.901 $\pm$ 0.25669 ^a
Plant Sample	200 μ M CuSO ₄	
	Control	
Leaf	1.20 $\pm$ 0.091 ^b	5.22 $\pm$ 0.868 ^a
Root	2.168 $\pm$ 0.190 ^b	3.75 $\pm$ 0.401 ^a

4.14.2 Root

In the cross-section of *V. inermis* root, the zones such as epidermis, root cortex, and vascular or central cylinder could be clearly distinguished. The outer single layered epidermis followed by 2-3 layers of hypodermis was composed of parenchymatous cells without intercellular space. Below the hypodermis, there is the aerenchymatous cortex, composed of loosely packed cells. The endodermis, the innermost layer of the cortex, is a layer of cells that surrounds the stele. The endodermis is followed by pericycle. The well-developed xylem elements and phloem forms the central cylinder. Although the number of phloem cells is low, xylem cells occupies a larger space of the central cylinder. Xylem vessels are round, oval or polygonal. There is no central parenchymatous pith as the xylem covers the entire central tissue regions of the root.

On exposure to 400 mM NaCl, the roots of *V. inermis* showed visible changes in epidermis and aerenchymatous cortex and the cells of these regions were seen ruptured. When compared to the control, the aerenchymatous cortical cells were smaller with smaller air cavities or even the air cavities were seen crushed. There were no depositions/inclusions visible in the cortical cells of the roots of plantlets treated with 400 mM NaCl. The central cylinder of the root was completely occupied by vascular tissues. The xylem vessels density was increased in the roots of plantlets exposed to 400 mM NaCl as compared to control. The phloroglucinol staining in the root showed the presence of lignified vascular cells and it was higher in the roots of 400 mM NaCl treated plantlets as compared with the roots of control plantlets (Fig. 38). The plantlets subjected to 200 μ M CuSO₄ exhibits similar results in the root anatomy. The epidermis and the

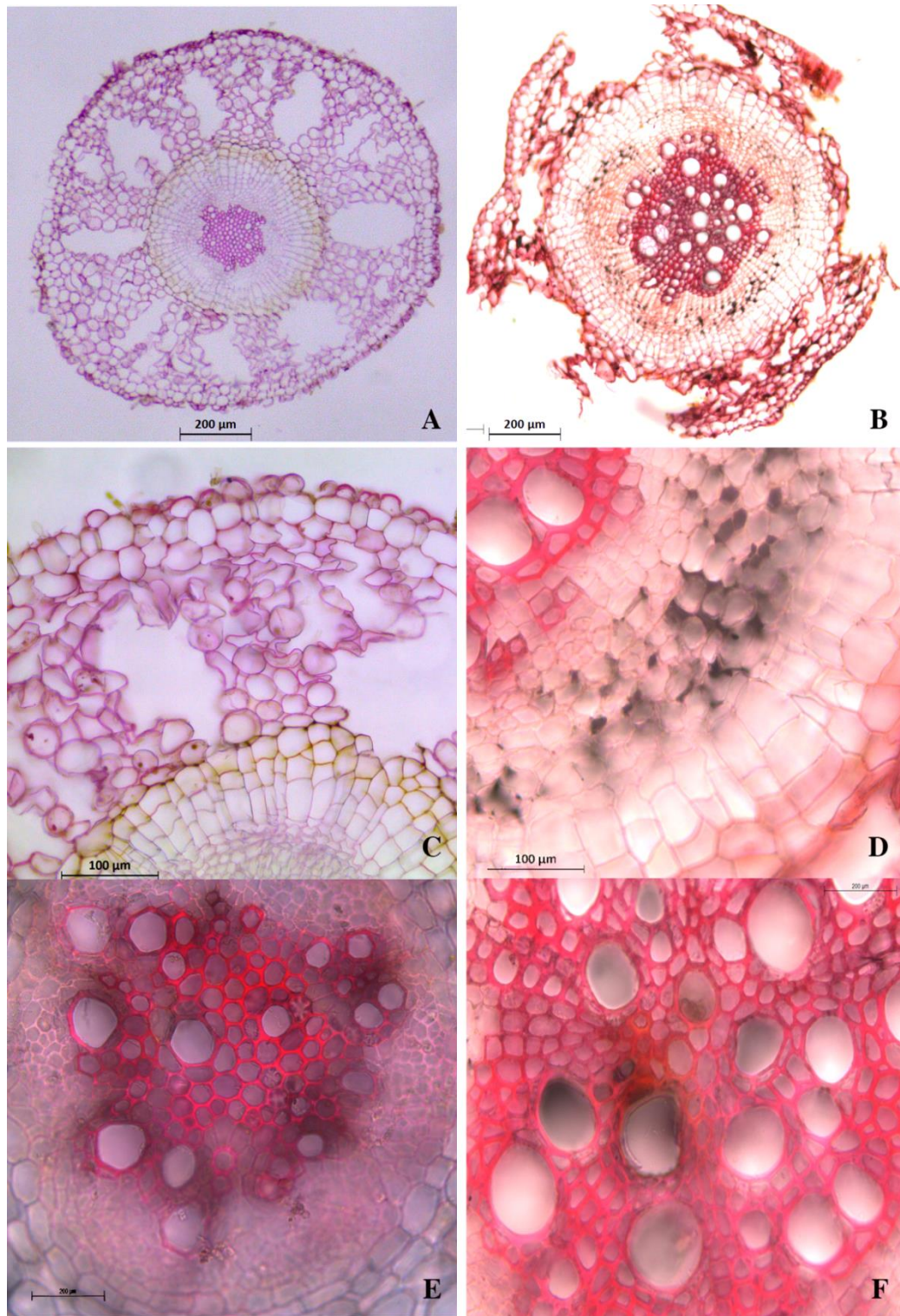


Figure 38. Anatomical variations found in the roots of *V. inermis* plantlets (cross-section view and vascular region) subjected to 400 mM NaCl. A- Control cross-section view, B- 400 mM NaCl cross-section view, C- Control hypodermis, D- 400 mM NaCl hypodermis, E- Control vascular region, F- 400 mM NaCl vascular region.

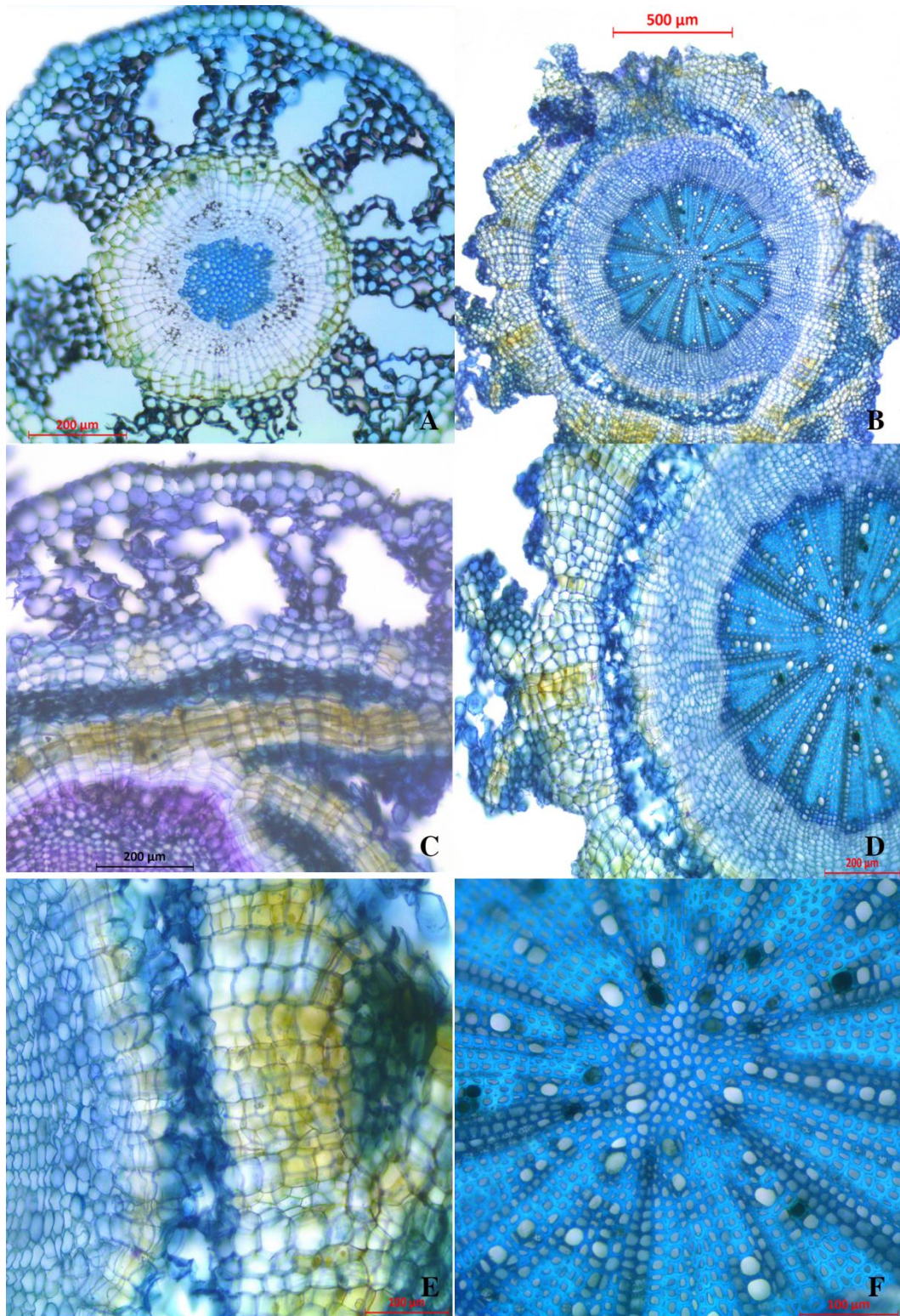


Figure 39. Anatomical variations found in the roots of *V. inermis* plantlets (cross-section view and vascular region) subjected to 200 μM CuSO_4 . A- Control cross-section view, B- 200 μM CuSO_4 cross-section view, C- Control hypodermis, D- 200 μM CuSO_4 hypodermis, E- 200 μM CuSO_4 endodermal region, F- 200 μM CuSO_4 vascular region.

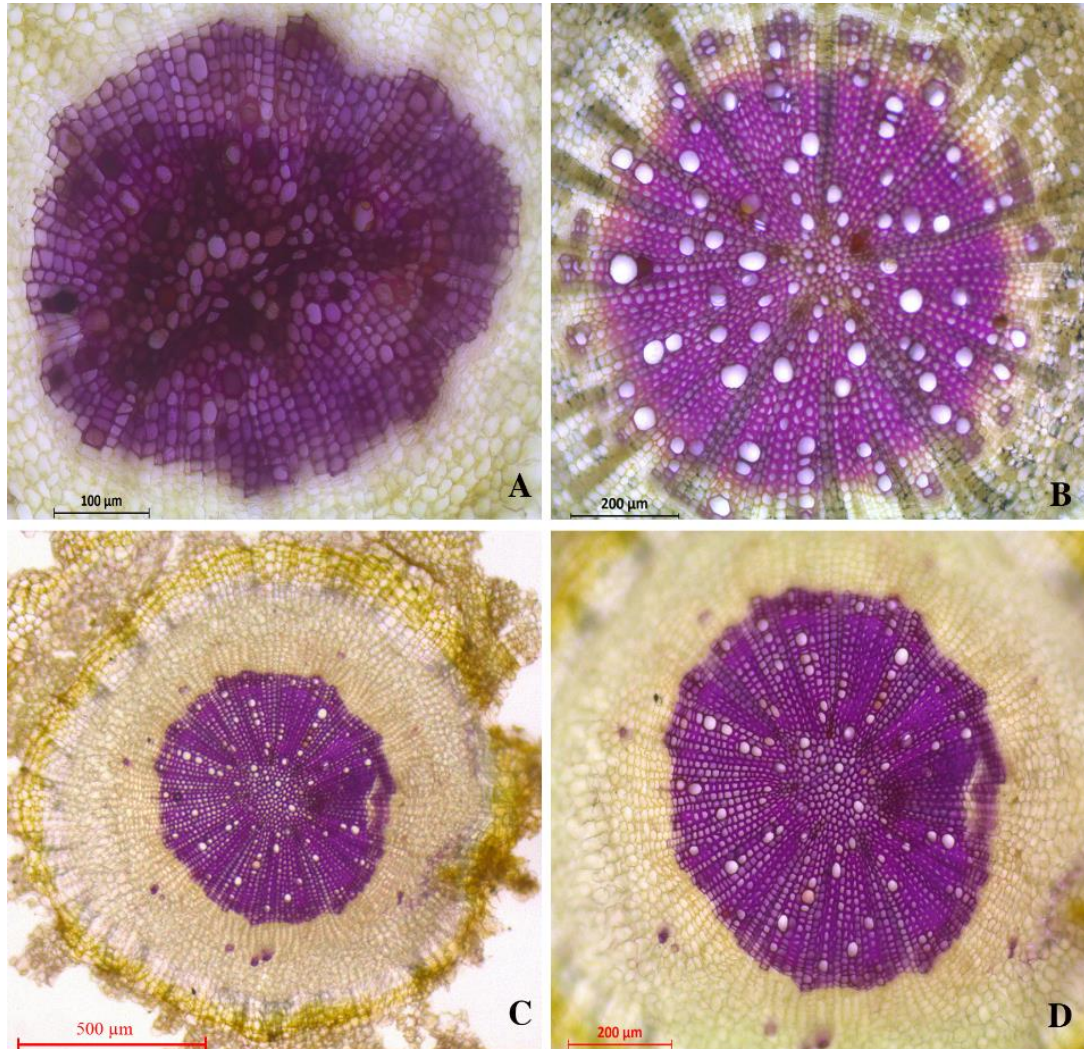


Figure 40. Phloroglucinol-HCl test for lignin in the roots of *V. inermis* plantlets subjected to 400 mM NaCl and 200 μ M CuSO₄. A- Control vascular region, B- 400 mM NaCl vascular region, C and D- 200 μ M CuSO₄ vascular region.

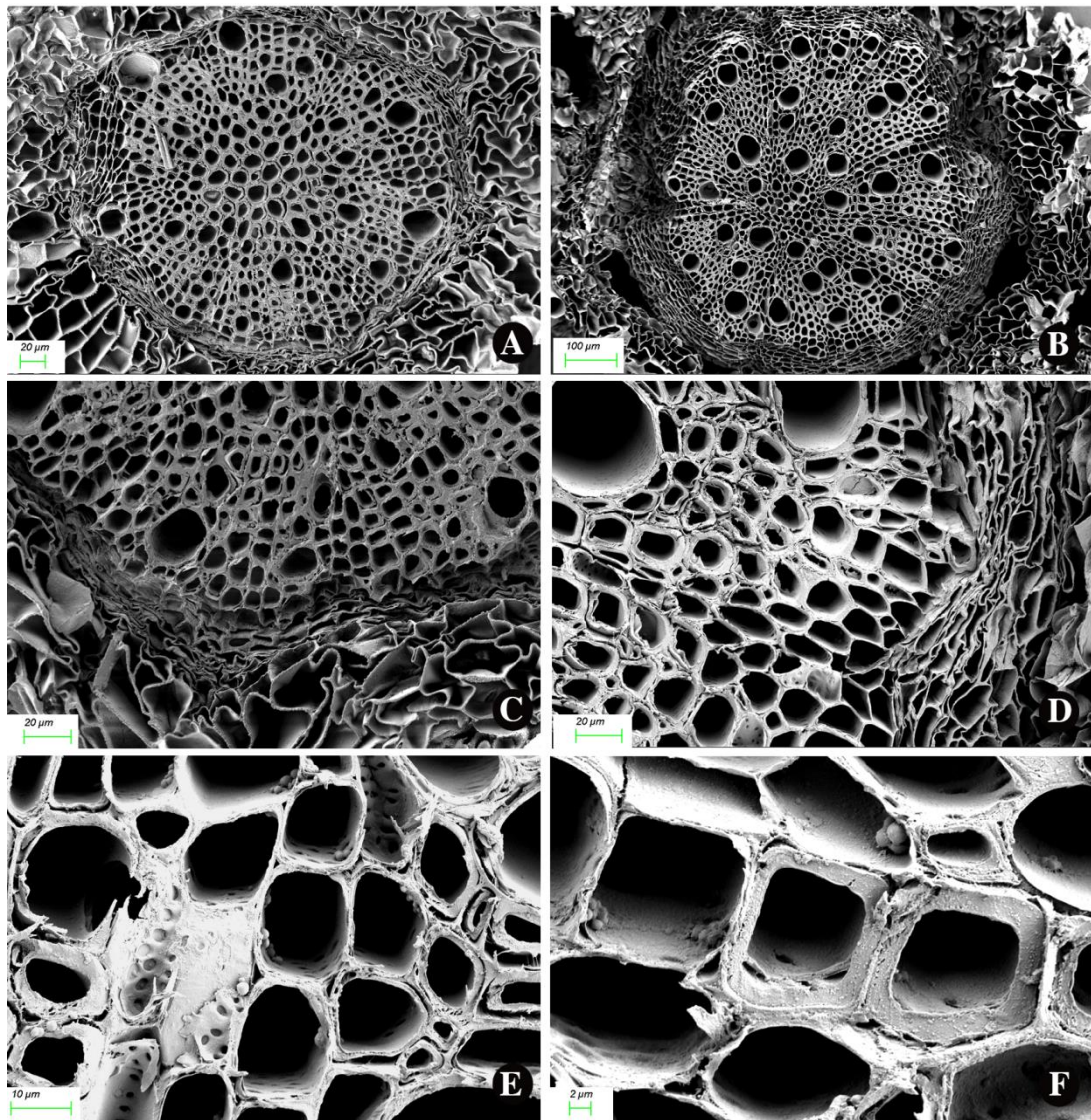


Figure 41. Scanning electron micrograph of *V. inermis* roots subjected to 400 mM NaCl. A- Control cross-section view, B- 400 mM NaCl cross-section view, C- Control vascular region, D- 400 mM NaCl vascular region, E- Blockage in vascular region in the roots of 400 mM NaCl, F- 400 mM NaCl xylem wall.

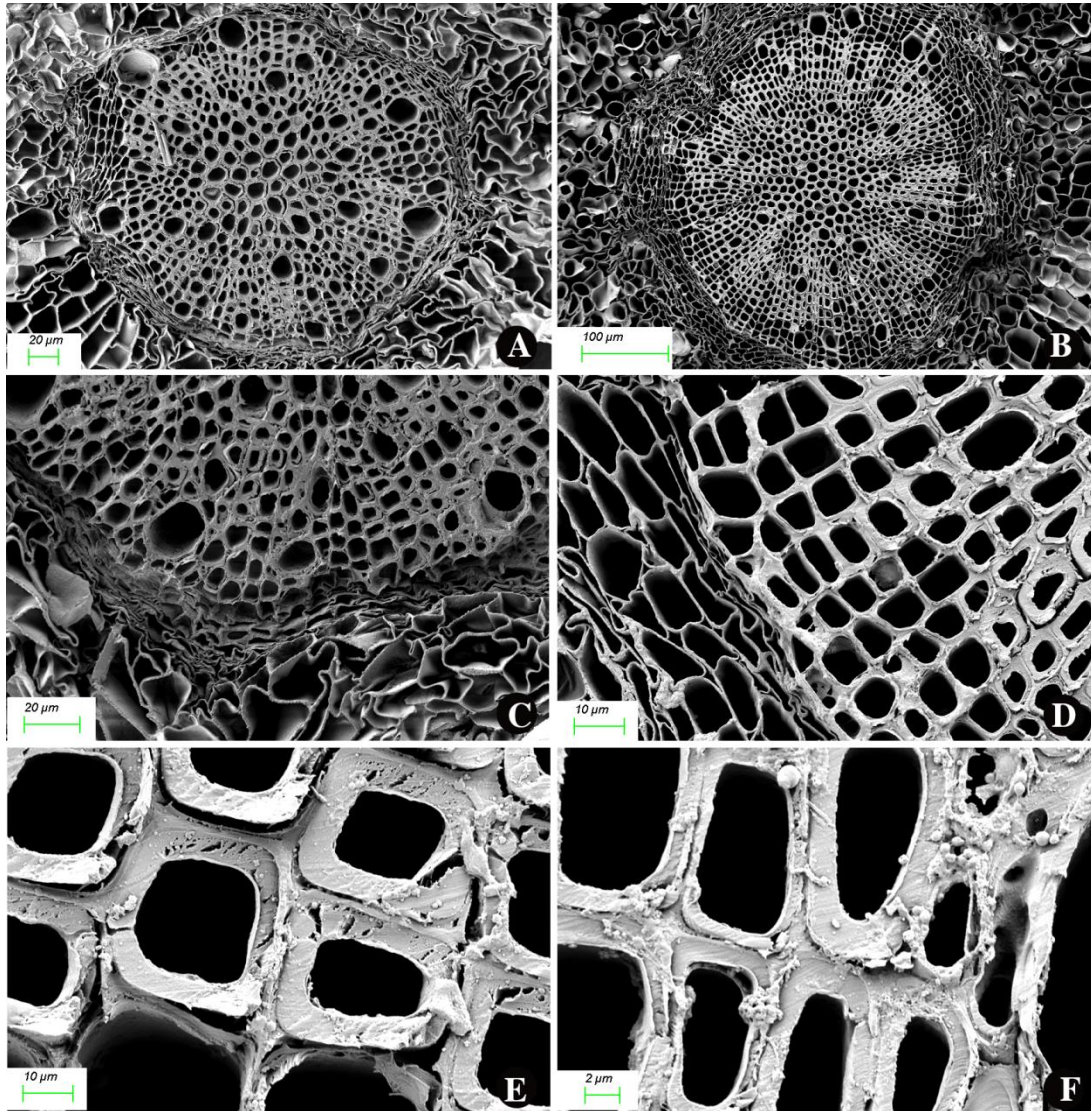


Figure 42. Scanning electron micrograph of *V. inermis* roots subjected to 200 μM CuSO_4 . A- Control cross-section view, B- 200 μM CuSO_4 cross-section view, C-Control vascular region, D- 200 μM CuSO_4 vascular region, E- Highly thickened xylem walls, F- xylem walls with deposition.

arrangement of the aerenchymatous cortex cells was distorted in response to 200 μM CuSO_4 (Fig. 39). Highly lignified cells were found in the cortical cylinder and it was observed with bright blue colour. The number of lignified cells was higher in the roots of 200 μM CuSO_4 treated plantlets as compared with roots of control plantlets. Xylem vessel density was increased in response to CuSO_4 treatment and the vessels were with smaller cavity than the control (Fig. 40).

In scanning electron microscopic images, the difference in the xylary elements of the vascular or central cylinder of treated (400 mM NaCl) and control was very clear. The increase in the vessel density in the roots of 400 mM NaCl exposed plantlets was very clear in the SEM images. The enhancement in the xylem wall thickness was noted in the roots of 400 mM NaCl exposed plantlets and the enhancement was 116% compared with the roots of control plantlets (Fig. 41). The exposure of plantlets to CuSO_4 resulted in similar kind of modifications. The increment in the xylem vessel number as well as, wall thickness was observed. Some of the xylem vessels had some depositions and it blocked the vessels completely. The shape of the cells including xylem vessels was totally distorted and cell walls were more thickened than control plantlets. The xylem wall had 73% increase in thickness than control plantlets (Fig. 42).

4.15 Secondary metabolites

4.15.1 LC-MS analysis

The methanolic extract of the various plant parts like leaves and roots of *V. inermis* treated with NaCl and CuSO_4 were subjected to the HR-LC MS analysis for identifying the non-volatile phytochemicals.

In the case of control leaves, total 29 compounds were identified at different retention times that ranged from 2.394 to 15.282 (Table 20). The high-resolution liquid chromatogram of both positive and negative ionization modes of the extract is shown in Figure 43. Phenols, terpenoids, alkaloids, flavonoids and glycosides are the major class of compounds identified through the analysis. Vanylglycol and chlorogenic acid were the only phenolic compounds detected in the control leaves. Sulochrin and oroxindin were the phenolic compounds observed in the CuSO₄ treated leaves. But in the case of leaves from NaCl treated plantlets, no phenolic compounds were identified by HR-LCMS analysis (Fig.44 and Table 21). Furthermore, highest number of non-volatile compounds were observed in CuSO₄ treated leaves (43 compounds) (Fig. 45 and Table 22), followed by the control (29 compounds) as well as NaCl treated leaves (18 compounds). Notoginsenoside A is a saponin which was detected in the NaCl treated leaves and no saponins were observed in both control and CuSO₄ treated leaves. Barbinine is an alkaloid compound found in NaCl treated leaves whereas, bebeerine has been the common alkaloid compound detected in control and CuSO₄ treated leaves. Similarly, Momilactone B is the common terpenoid compound found in control and NaCl treated leaves and (+)-Sophorol is the flavonoid compound observed in both control and CuSO₄ treated leaves.

The presence of non-volatile compounds in the roots of *V. inermis* exposed to NaCl and CuSO₄ were also detected by the HR-LC MS analysis. Total 28 non-volatile phytochemicals were identified in the control root at different retention times (Fig. 46 and Table 23). They were ranging from

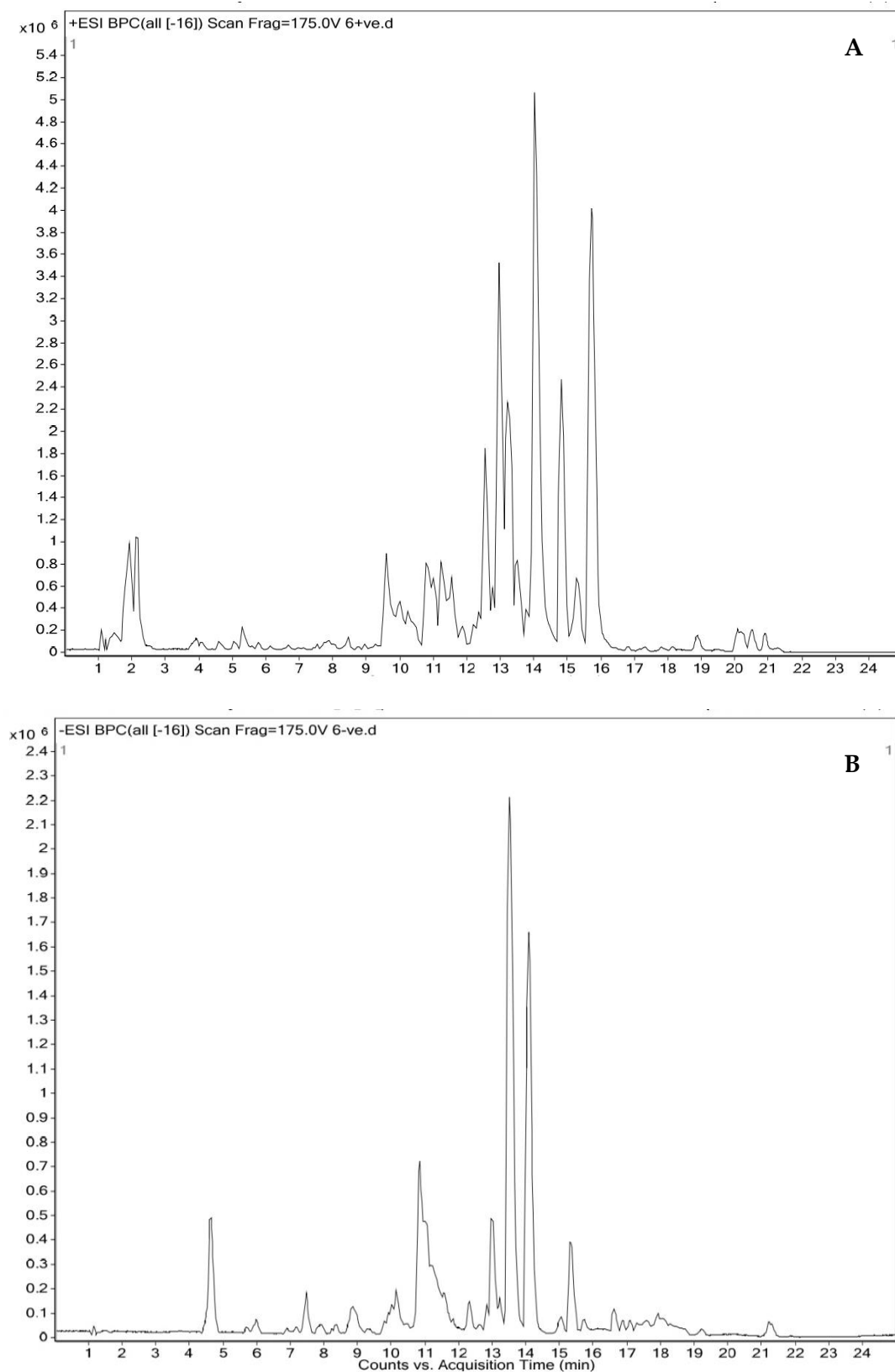


Figure 43. HR-LCMS chromatogram (A-Positive ESI and B-Negative ESI) of methanolic extract of *V. inermis* control leaves.

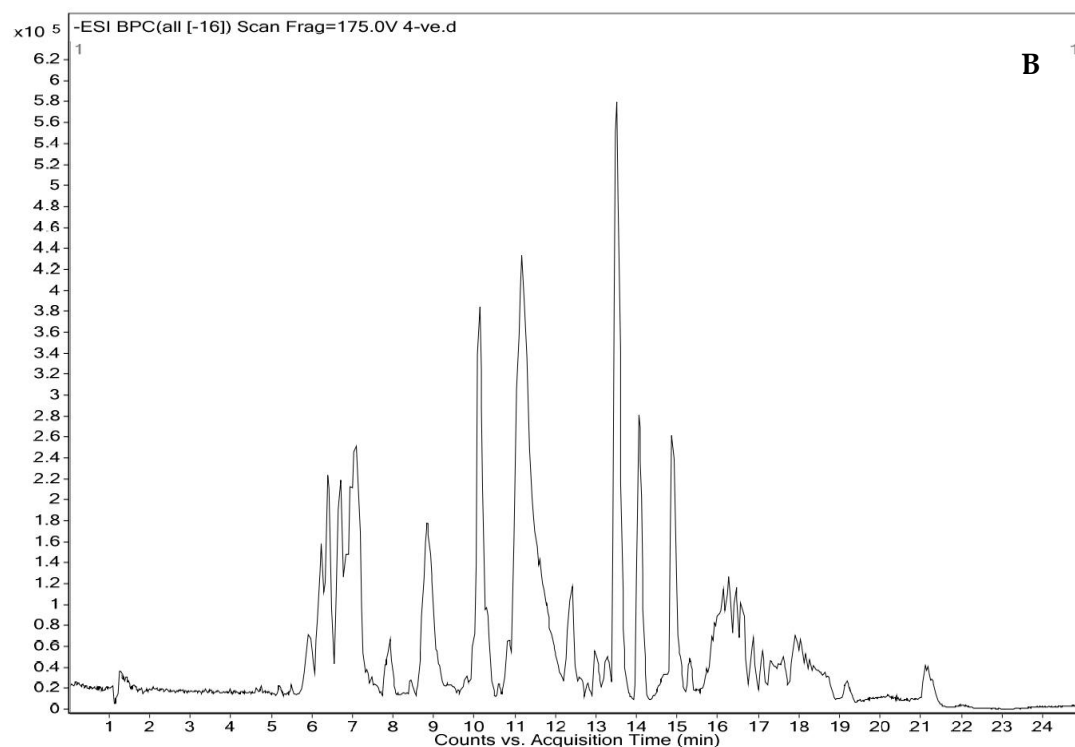
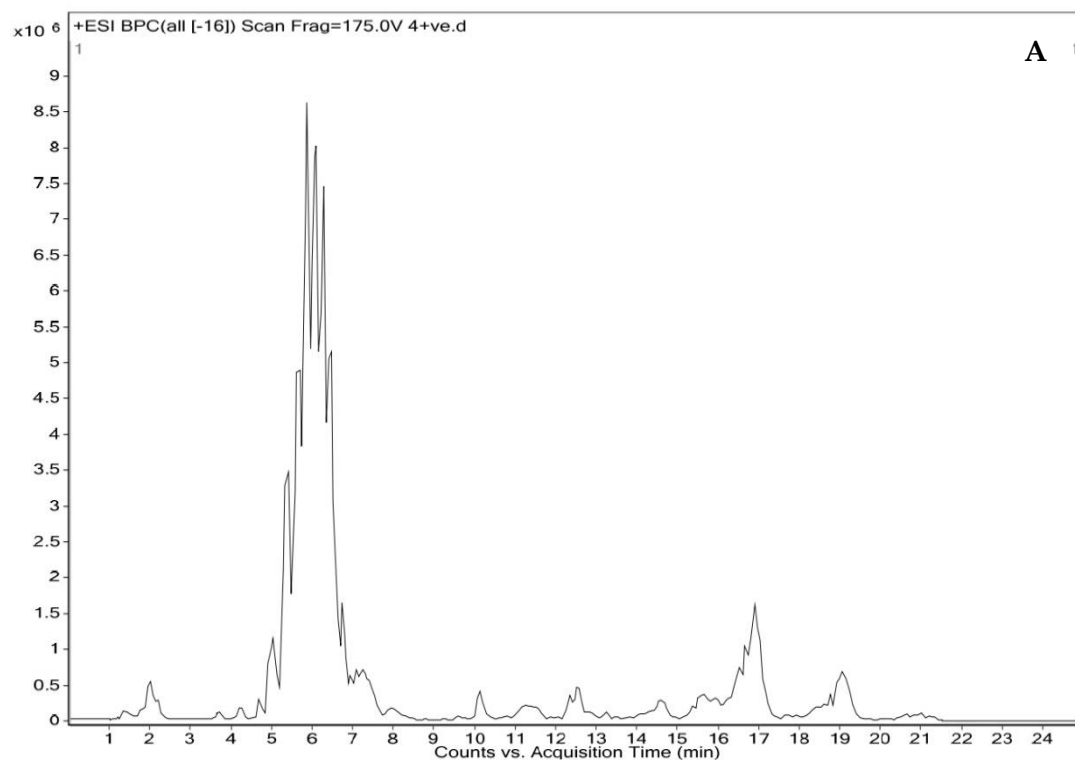


Figure 44. HR-LCMS chromatogram (A-Positive ESI and B-Negative ESI) of methanolic extract from the leaves of *V. inermis* plantlets exposed to 400 mM NaCl on 20d.

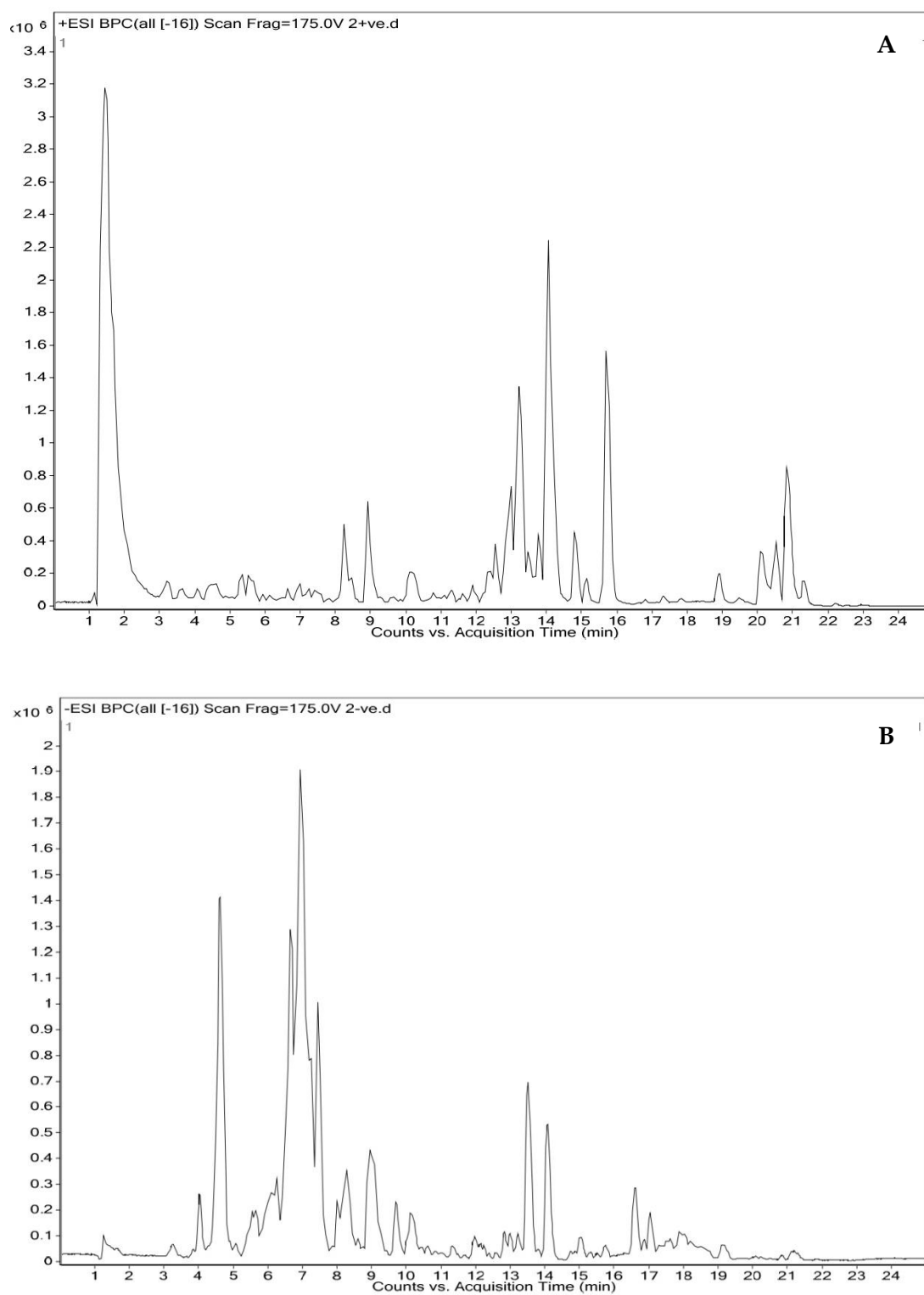


Figure 45. HR-LCMS chromatogram (A-Positive ESI and B-Negative ESI) of methanolic extract from the leaves of *V. inermis* plantlets exposed to 200 μM CuSO_4 on 20d.

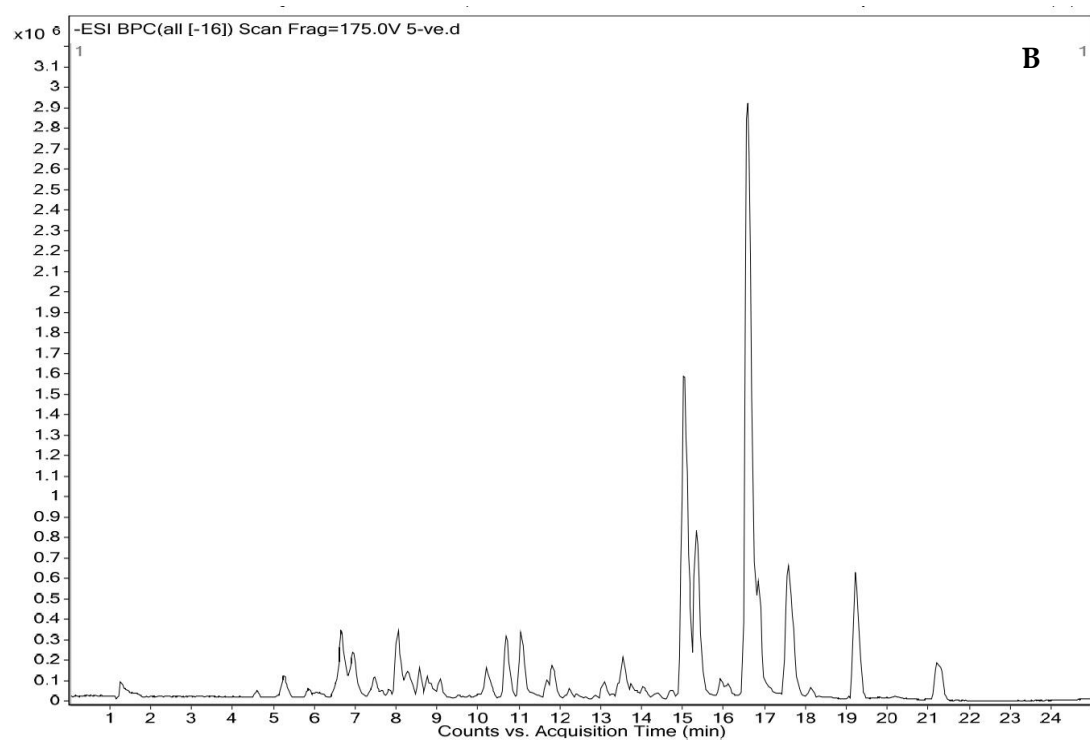
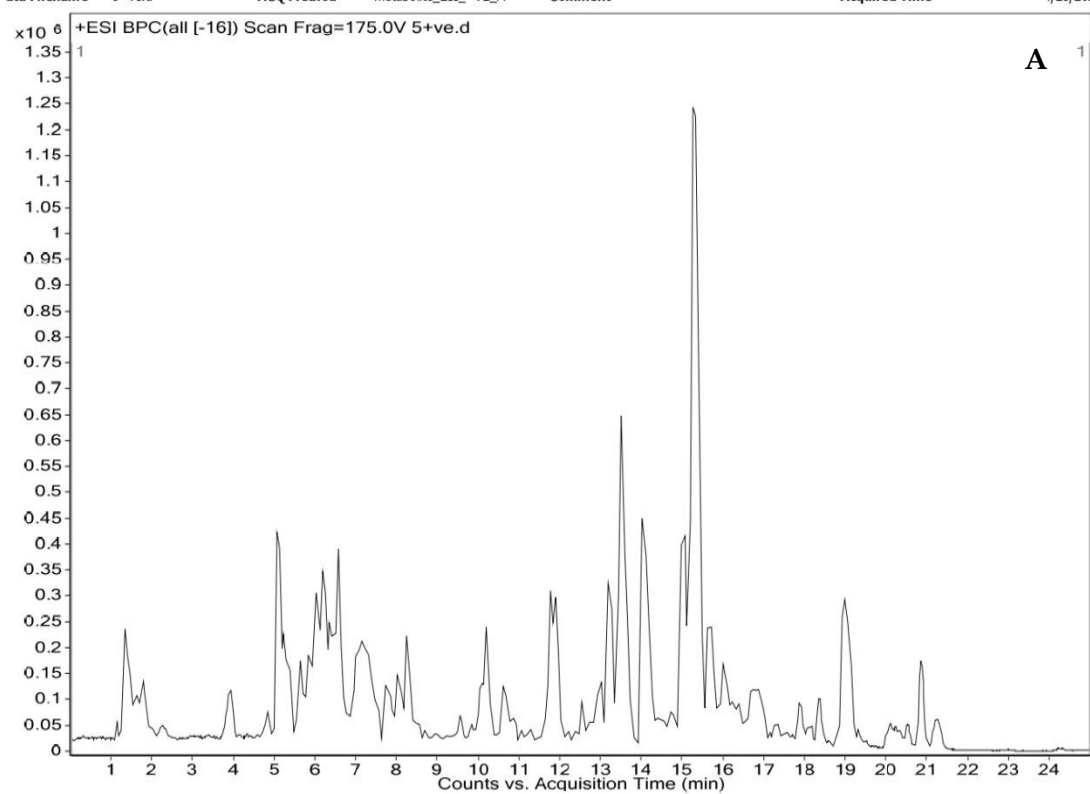


Figure 46. HR-LCMS chromatogram (A-Positive ESI and B-Negative ESI) of methanolic extract of *V. inermis* control roots.

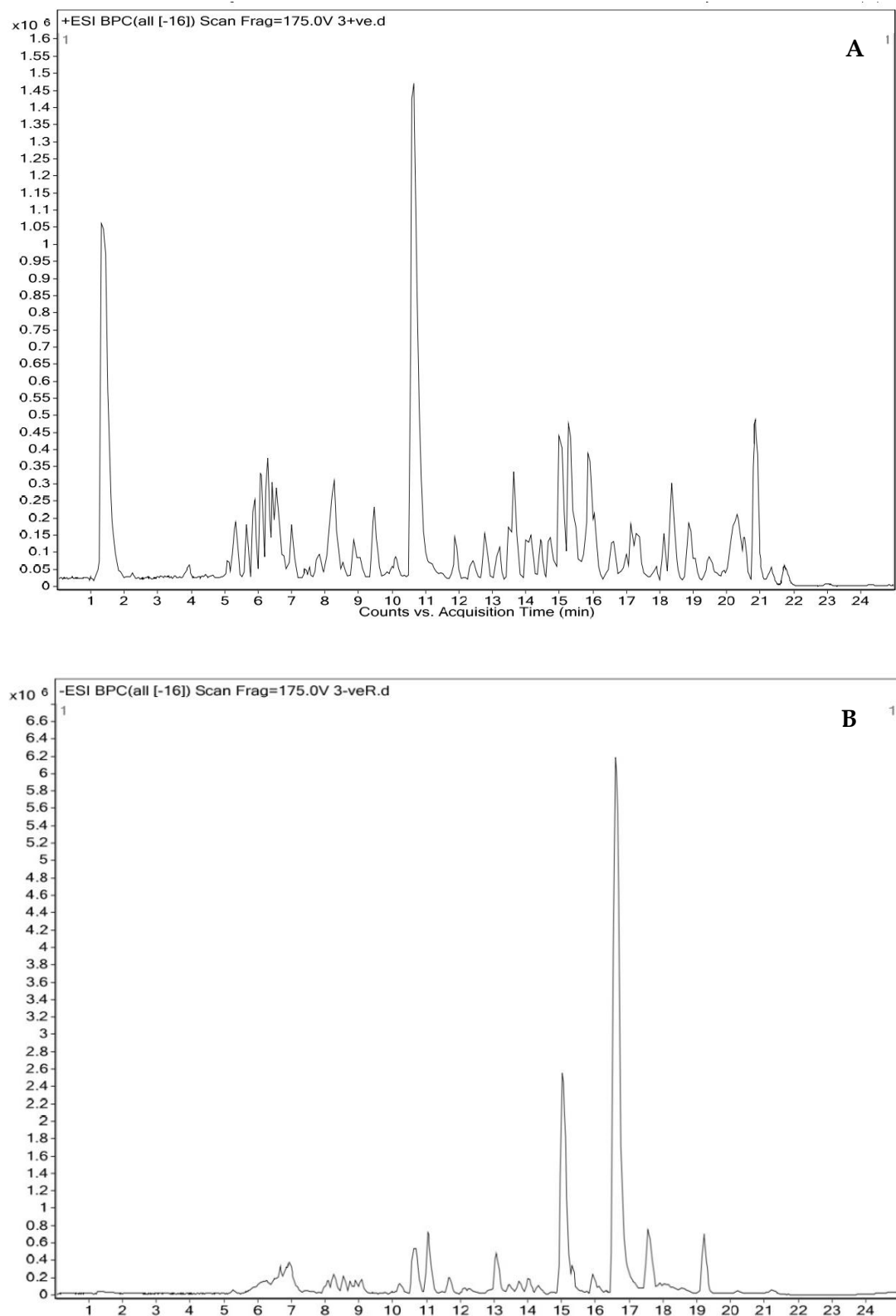


Figure 47. HR-LCMS chromatogram (A-Positive ESI and B-Negative ESI) of methanolic extract from the roots of *V. inermis* plantlets exposed to 400 mM NaCl on 20d.

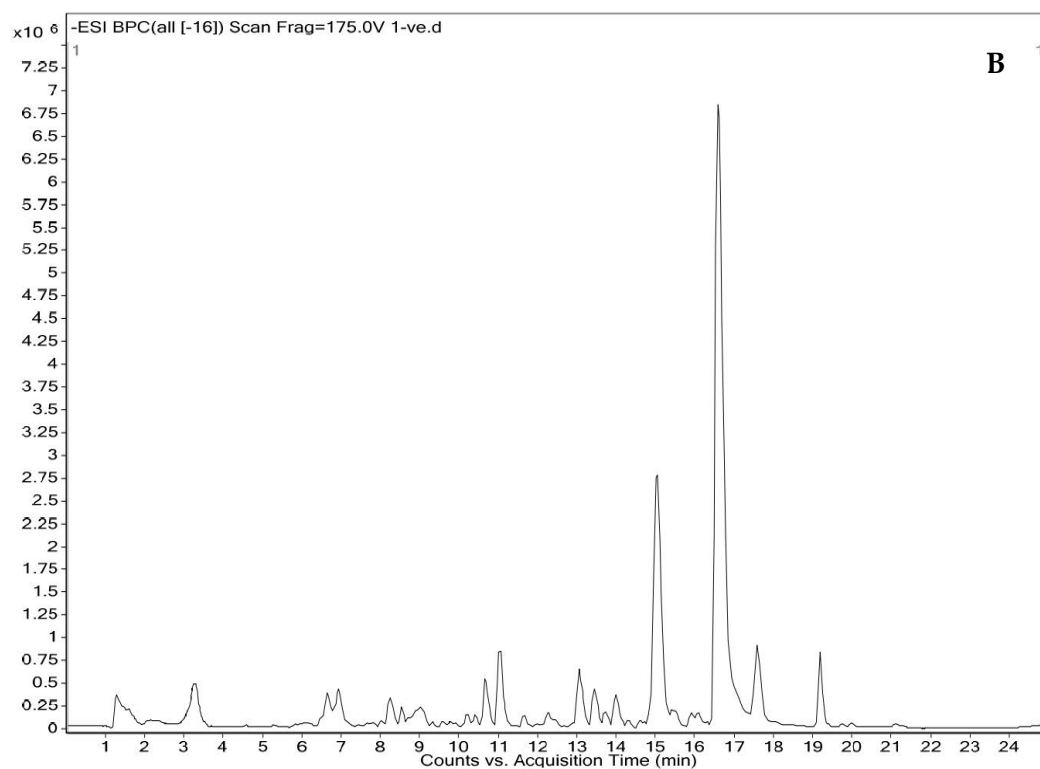
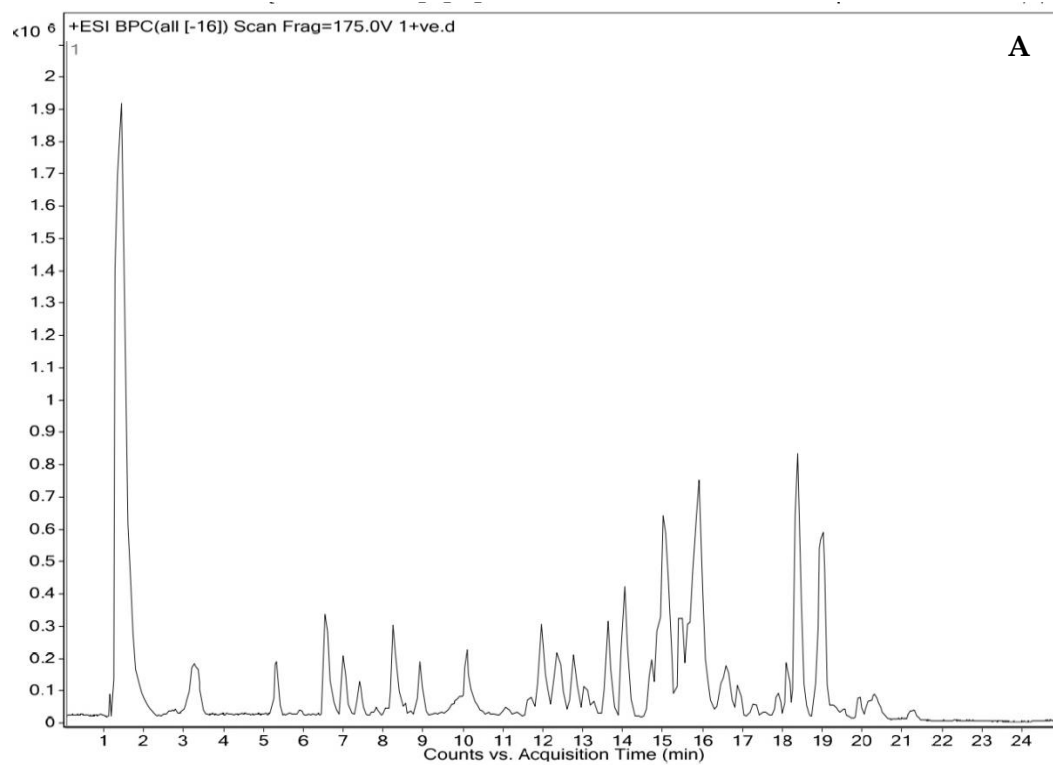


Figure 48. HR-LCMS chromatogram (A-Positive ESI and B-Negative ESI) of methanolic extract from the roots of *V. inermis* plantlets exposed to 200 μ M CuSO_4 on 20d.

Table 20. Phytochemical constituents detected in the methanolic extract from the leaves of *V. inermis* control plantlets identified through HR LC-MS.

Sl/No	RT	Compound	Molecular formula	Class	Molecular mass	<i>m/z</i>
1	2.394	Vanylglycol	C ₉ H ₁₂ O ₄	Phenol	184.0726	185.0799
2	4.44	(+)-Plicamine	C ₂₆ H ₂₆ N ₂ O ₆	Alkaloids	462.1777	507.176
3	4.98	Dioscoretine	C ₁₃ H ₂₃ N O ₃	Alkaloid	241.1685	264.1579
4	5.199	Feruloylputrescine	C ₁₄ H ₂₀ N ₂ O ₃	Coumarin	264.1467	265.1544
5	5.593	Chlorogenic acid	C ₁₆ H ₁₈ O ₉	Phenol	354.0942	399.0928
6	6.066	Gniditrin	C ₃₇ H ₄₂ O ₁₀	Terpenoid	646.2888	691.287
7	7.074	Trichilin A	C ₃₅ H ₄₆ O ₁₃	Terpenoid	674.2845	673.2764
8	8.39	Bufotenine O-glucoside	C ₁₈ H ₂₆ N ₂ O ₆	Glycoside	366.1794	389.1687
9	9.3	(+)-Sophorol	C ₁₆ H ₁₂ O ₆	Flavonoid	300.0618	301.0691
10	9.436	Licoagrodione	C ₂₀ H ₂₀ O ₆	Flavonoid	356.124	357.1312
11	9.836	Vernolepin	C ₁₅ H ₁₆ O ₅	Terpenoid	276.1005	299.0897
12	10.03	Baicalein	C ₁₅ H ₁₀ O ₅	Flavonoid	270.0558	269.0485
13	10.064	Coriandrone E	C ₁₃ H ₁₂ O ₅	Coumarin	248.0695	271.0587
14	10.179	Isokaempferide	C ₁₆ H ₁₂ O ₆	Flavonoid	300.0666	299.059
15	12.501	Vignatic acid A	C ₃₀ H ₃₉ N ₃ O ₇	Amino acid	553.2863	554.2933
16	12.507	Glaucarubol 15-O-beta-D-glucopyranoside	C ₂₆ H ₃₈ O ₁₃	Terpenoid	558.2415	559.2487
17	12.771	Cerbertin	C ₃₂ H ₄₄ O ₁₁	Phytosterol	604.2833	605.2905
18	12.97	Bebeerine	C ₃₆ H ₃₈ N ₂ O ₆	Alkaloid	594.2651	617.2543
19	13.217	Caryoptin	C ₂₆ H ₃₆ O ₉	Terpenoid	492.2334	515.2226

Sl/No	RT	Compound	Molecular formula	Class	Molecular mass	<i>m/z</i>
20	13.273	Chamazulene	C ₁₄ H ₁₆	Terpenoid	184.124	185.1312
21	13.504	adonitoxin	C ₂₉ H ₄₂ O ₁₀	Glycoside	550.2755	551.2828
22	13.558	Calotropin	C ₂₉ H ₄₀ O ₉	Glycoside	532.2643	533.2715
23	13.763	Nigakilactone B	C ₂₂ H ₃₂ O ₆	Terpenoid	392.2198	415.209
24	14.113	Cimifugin	C ₁₆ H ₁₈ O ₆	Glycoside	306.1108	329.0999
25	14.222	Tinyatoxin	C ₃₆ H ₃₈ O ₈	Terpenoid	598.2598	643.2597
26	14.95	Momilactone B	C ₂₀ H ₂₆ O ₄	Terpenoid	330.1867	329.1794
27	15.15	Adouetine Z	C ₄₂ H ₄₅ N ₅ O ₅	Alkaloid	699.3432	700.3504
28	15.21	Vernolepin	C ₁₅ H ₁₆ O ₅	Terpenoid	276.1006	299.0898
29	15.282	Limonoate	C ₂₆ H ₃₄ O ₁₀	Carboxylic acid	506.2183	507.2257

Table 21. Phytochemical constituents detected in the methanolic extract from the leaves of *V. inermis* plantlets exposed to 400 mM NaCl on 20d identified through HR LC-MS.

Sl/No	RT	Compound	Molecular formula	Class	Molecular mass	<i>m/z</i>
1	5.811	Phenylethyl primeveroside	C ₁₉ H ₂₈ O ₁₀	Glycoside	416.1625	415.1644
2	5.866	Auriculine	C ₃₁ H ₄₅ N O ₈	Carbohydrate	559.3182	560.3254
3	5.915	3b,6a-Dihydroxy-alpha-ionol 9- [apiosyl-(1->6)-glucoside]	C ₂₄ H ₄₀ O ₁₂	Glycoside	520.2471	521.2544
4	6.161	Rodiasine	C ₃₈ H ₄₂ N ₂ O ₆	Alkaloid	622.2997	621.2946
5	6.267	Hexaprenyl diphosphate	C ₃₀ H ₅₂ O ₇ P ₂	Terpenoid	586.3179	587.3253
6	6.258	Rhodexin A	C ₂₉ H ₄₄ O ₉	Glycoside	536.2987	581.299
7	6.305	Barbinine	C ₃₆ H ₄₆ N ₂ O ₁₀	Alkaloid	666.3263	665.321
8	6.761	Quercetin-4'-glucuronide	C ₂₁ H ₁₈ O ₁₃	Glycoside	478.0791	477.0719
9	6.932	Notoginsenoside A	C ₅₄ H ₉₂ O ₂₄	Saponin	1124.6027	1123.5976
10	6.987	Ginsenoside Rb1	C ₅₄ H ₉₂ O ₂₃	Glycoside	1108.6077	1167.6241
11	8.409	Chamaemeloside	C ₂₇ H ₂₈ O ₁₄	Glycoside	576.1534	621.1516
12	8.796	Isokaempferide	C ₁₆ H ₁₂ O ₆	Flavonoid	300.0663	299.059
13	10.031	Baicalein	C ₁₅ H ₁₀ O ₅	Flavonoid	270.0558	269.0484
14	10.376	Cinn cassiol D2	C ₂₀ H ₃₂ O ₆	Terpenoid	368.2234	367.2161
15	10.38	Cirsiliol	C ₁₇ H ₁₄ O ₇	Flavonoid	330.0776	329.0703
16	14.054	Tinyatoxin	C ₃₆ H ₃₈ O ₈	Terpenoid	598.2588	643.2592
17	16.627	Momilactone B	C ₂₀ H ₂₆ O ₄	Terpenoid	330.1869	329.1796
18	16.901	6-Geranylgeranyl 8'-methyl 6,8'-diapocarotene-6,8'-dioate	C ₄₃ H ₆₀ O ₄	Terpenoid	640.4375	663.4268

Table 22. Phytochemical constituents detected in the methanolic extract from the leaves of *V. inermis* plantlets exposed to 200 μ M CuSO₄ on 20d identified through HR LC-MS.

Sl/No	RT	Compound	Molecular formula	Class	Molecular mass	<i>m/z</i>
1	1.323	Sulochrin	C ₁₇ H ₁₆ O ₇	Phenol	332.089	377.0892
2	1.373	Retronecine	C ₈ H ₁₃ N O ₂	Alkaloid	155.0941	156.1013
3	3.891	Paeonolide	C ₂₀ H ₂₈ O ₁₂	Glycoside	460.1649	505.1607
4	4.33	(+)-Plicamine	C ₂₆ H ₂₆ N ₂ O ₆	Alkaloid	462.1784	507.1766
5	4.376	Convoline	C ₁₆ H ₂₁ N O ₅	Alkaloid	307.1421	330.1314
6	4.642	4,7-Didehydroneophysalin B	C ₂₈ H ₂₈ O ₉	Phytosterol	508.1761	531.1652
7	5.157	Gnetin A	C ₂₈ H ₂₂ O ₆	Polyphenol	454.1427	455.1495
8	5.409	Millettone	C ₂₂ H ₁₈ O ₆	Flavonoid	378.1103	437.1262
9	5.644	Glisoflavanone	C ₂₅ H ₂₈ O ₆	Flavonoid	424.1879	423.1823
10	6.021	Isoacteoside	C ₂₉ H ₃₆ O ₁₅	Glycoside	624.2111	623.204
11	6.032	Lavandulifolioside	C ₃₄ H ₄₄ O ₁₉	Carbohydrate	756.2538	755.2465
12	6.284	Citbismine E	C ₃₆ H ₃₄ N ₂ O ₁₁	Alkaloid	670.2172	669.2101
13	6.598	Catechin 3',5-diglucoside	C ₂₇ H ₃₄ O ₁₆	Flavonoid	614.1807	659.181
14	7.451	Trichilin A	C ₃₅ H ₄₆ O ₁₃	Terpenoid	674.2855	673.2782
15	7.541	Nomilinic acid 17-glucoside	C ₃₄ H ₄₈ O ₁₆	Glycoside	712.3011	757.2995
16	7.708	Gibberellin A37 glucosyl ester	C ₂₆ H ₃₆ O ₁₀	Terpenoid	508.2366	507.2289
17	8.024	Diosbulbinoside F	C ₂₆ H ₃₄ O ₁₂	Terpenoid	538.2111	583.2094
18	8.091	6''-(4-Carboxy-3-hydroxy-3-methylbutanoyl) hyperin	C ₂₇ H ₂₈ O ₁₆	Flavonoid	608.135	609.1421

Sl/No	RT	Compound	Molecular formula	Class	Molecular mass	<i>m/z</i>
19	8.247	Eridictiol	C ₂₂ H ₂₀ O ₁₂	Glycoside	476.0927	477.1
20	8.409	Bufotenine O-glucoside	C ₁₈ H ₂₆ N ₂ O ₆	Glycoside	366.179	389.1681
21	8.571	Hemigossypolone	C ₁₅ H ₁₄ O ₅	Terpenoid	274.0847	297.0738
22	8.921	7,3'-Di-O-methylisoorientin	C ₂₃ H ₂₄ O ₁₁	Flavonoid	476.129	477.136
23	9.022	Loquatoside	C ₂₀ H ₂₂ O ₁₁	Glycoside	438.1162	461.1052
24	9.027	Oroxindin	C ₂₂ H ₂₀ O ₁₁	Phenol	460.1057	459.0985
25	9.695	Corchorifatty acid F	C ₁₈ H ₃₂ O ₅	Fatty acid	328.2289	327.2215
26	10.088	Coriandrone E	C ₁₃ H ₁₂ O ₅	Isocoumarins	248.0697	271.0588
27	10.096	Gravacridonetriol	C ₁₉ H ₁₉ N O ₆	Glycoside	357.1214	380.1107
28	10.1	(+)-Sophorol	C ₁₆ H ₁₂ O ₆	Flavonoid	300.0617	301.0688
29	10.147	Isokaempferide	C ₁₆ H ₁₂ O ₆	Flavonoid	300.0665	299.0592
30	10.223	9,10-Dihydroxy-12,13-epoxyoctadecanoate	C ₁₈ H ₃₄ O ₅	Fatty acid	330.2441	329.2368
31	12.406	Morindone	C ₁₅ H ₁₀ O ₅	Anthraquinone	270.0516	269.0443
32	12.565	Vignatic acid A	C ₃₀ H ₃₉ N ₃ O ₇	Peptide	553.286	554.2932
33	13.01	Bebeerine	C ₃₆ H ₃₈ N ₂ O ₆	Alkaloid	594.2646	617.254
34	13.157	Terminaline	C ₂₃ H ₄₁ N O ₂	Phytosterol	363.3113	364.3185
35	13.23	Caryoptin	C ₂₆ H ₃₆ O ₉	Terpenoid	492.2332	515.2224
36	13.485	Bakkenolide C	C ₂₀ H ₂₈ O ₅	Terpene	348.1936	371.1826
37	13.494	Adonitoxin	C ₂₉ H ₄₂ O ₁₀	Glycoside	550.275	551.2824
38	13.498	Di-n-pentyl phthalate	C ₁₈ H ₂₆ O ₄	Carboxylic ester	306.1839	329.1726

Sl/No	RT	Compound	Molecular formula	Class	Molecular mass	<i>m/z</i>
39	13.912	Tinyatoxin	C ₃₆ H ₃₈ O ₈	Terpene	598.2595	643.2598
40	14.075	Cimifugin	C ₁₆ H ₁₈ O ₆	Coumarins	306.1108	329.0999
41	15.116	Adouetine Z	C ₄₂ H ₄₅ N ₅ O ₅	Alkaloid	699.3435	700.3507
42	15.304	Limonoate	C ₂₆ H ₃₄ O ₁₀	Carboxylic acid	506.218	507.225
43	19.377	Neoporrigenin B	C ₂₇ H ₄₂ O ₅	Terpenoid	446.2981	447.3054

Table 23. Phytochemical constituents detected in the methanolic extract from the roots of *V. inermis* control plantlets identified through HR LC-MS.

Sl/No	RT	Compound	Molecular formula	Class	Molecular mass	<i>m/z</i>
1	1.418	Retronecine	C ₈ H ₁₃ N O ₂	Alkaloid	155.0942	156.1014
2	1.638	8-Hydroxyadenine	C ₅ H ₅ N ₅ O	Alkaloid	151.049	152.0565
3	5.081	Feruloylputrescine	C ₁₄ H ₂₀ N ₂ O ₃	Coumarins	264.1461	265.1535
4	6.535	Gibberellin A102	C ₂₀ H ₂₆ O ₇	Terpenoid	378.1681	401.1574
5	6.58	Isoacteoside	C ₂₉ H ₃₆ O ₁₅	Glycosides	624.2108	623.2036
6	6.962	Catechin 3',5-diglucoside	C ₂₇ H ₃₄ O ₁₆	Flavonoids	614.1808	659.1792
7	7.37	Osmanthuside B	C ₂₉ H ₃₆ O ₁₃	Glycosides	592.2208	637.2191
8	8.043	Cancentrine	C ₃₆ H ₃₄ N ₂ O ₇	Alkaloid	606.2369	651.2351
9	8.27	Diosmetin 7-O-beta-D-glucuronopyranoside	C ₂₂ H ₂₀ O ₁₂	Glycoside	476.0927	477.0999
10	8.612	Steviolbioside	C ₃₂ H ₅₀ O ₁₃	Diterpenoid	642.331	641.3237
11	9.075	Dehydroisoandrosterone 3-glucuronide	C ₂₅ H ₃₆ O ₈	Steroid	464.2459	509.2443
12	9.775	Quassinol	C ₂₀ H ₂₄ O ₆	Terpenoid	360.156	361.1626
13	9.93	Vernolepin	C ₁₅ H ₁₆ O ₅	Sesquiterpene	276.1005	299.0897
14	10.059	Coriandrone E	C ₁₃ H ₁₂ O ₅	Benzopyrans	248.0692	271.0582
15	10.126	Gravacridonetriol	C ₁₉ H ₁₉ N O ₆	Acridones	357.1211	380.1102
16	10.804	(+)-Sophorol	C ₁₆ H ₁₂ O ₆	Flavonoids	300.0616	301.0689
17	11.1	3-α(S)-Strictosidine	C ₂₇ H ₃₄ N ₂ O ₉	Alkaloid	530.2313	529.2259

Sl/No	RT	Compound	Molecular formula	Class	Molecular mass	<i>m/z</i>
18	12.575	Vignatic acid A	C ₃₀ H ₃₉ N ₃ O ₇	Amino acid	553.286	554.2932
19	12.998	Momilactone B	C ₂₀ H ₂₆ O ₄	Terpenoid	330.1865	329.1792
20	13.215	Caryoptin	C ₂₆ H ₃₆ O ₉	Terpenoid	492.2332	515.2224
21	13.473	Butyl 2-aminobenzoate	C ₁₁ H ₁₅ N O ₂	Esters	193.1093	194.1166
22	13.687	Nigakilactone B	C ₂₂ H ₃₂ O ₆	Terpenoid	392.2199	415.2091
23	14.131	Cimifugin	C ₁₆ H ₁₈ O ₆	Ketone	306.1109	329.1
24	15.287	Limonoate	C ₂₆ H ₃₄ O ₁₀	Terpenoid	506.2182	507.2255
25	15.288	Physalin L	C ₂₈ H ₃₂ O ₁₀	Steroid	528.2	529.2074
26	15.497	Sterebin G	C ₂₀ H ₃₄ O ₅	Terpenoid	354.2408	377.2299
27	17.894	Oryzalic acid B	C ₂₀ H ₃₀ O ₅	Diterpene	350.2093	373.1986
28	19.211	Ursolic acid	C ₃₀ H ₄₈ O ₃	Triterpenoid	456.3655	455.3583

Table 24. Phytochemical constituents detected in the methanolic extract from the roots of *V. inermis* plantlets exposed to 400 mM NaCl on 20d identified through HR LC-MS.

Sl/No	RT	Compound	Molecular formula	Class	Molecular mass	<i>m/z</i>
1	1.397	Retronecine	C ₈ H ₁₃ N O ₂	Alkaloid	155.094	156.1013
2	1.684	Malic acid	C ₄ H ₆ O ₅	Carboxylic acid	134.0221	133.0148
3	2.887	Gallic acid	C ₇ H ₆ O ₅	Phenolic acid	168.0425	169.015
4	5.161	Feruloylputrescine	C ₁₄ H ₂₀ N ₂ O ₃	Coumarins	264.1457	265.1534
5	5.58	5,7-Dihydroxychromone	C ₉ H ₆ O ₄	Coumarin	178.0273	177.0199
6	5.805	Caffeic acid	C ₉ H ₈ O ₄	Polyphenol	180.0428	179.0356
7	6.548	Gibberellin A102	C ₂₀ H ₂₆ O ₇	Terpenoid	378.1677	401.1574
8	6.58	Fluridone	C ₁₉ H ₁₄ F ₃ N O	Pyridines	329.1008	330.1075
9	7.054	Catechin3',5-diglucoside	C ₂₇ H ₃₄ O ₁₆	Flavonoids	614.1802	659.1784
10	6.694	Isoacteoside	C ₂₉ H ₃₆ O ₁₅	Glycosides	624.2093	623.202
11	7.671	Ferulic acid	C ₁₀ H ₁₀ O ₄	Phenol	194.0589	193.0516
12	7.743	Gibberellin A39	C ₂₀ H ₂₆ O ₈	Terpenoid	394.1629	417.1521
13	8.221	Diosmetin 7-O-beta-D-glucuronopyranoside	C ₂₂ H ₂₀ O ₁₂	Flavonoids	476.0994	475.0922
14	8.278	Oolonghomobisflavan A	C ₄₅ H ₃₆ O ₂₂	Polyphenol	928.1729	973.1709
15	8.631	Steviolbioside	C ₃₂ H ₅₀ O ₁₃	Glycosides	642.3298	641.3226
16	8.693	Scutianine F	C ₃₈ H ₄₅ N ₅ O ₅	Alkaloid	651.3464	674.3356
17	8.781	Gniditrin	C ₃₇ H ₄₂ O ₁₀	Terpenoid	646.2777	691.2781
18	9.034	Dehydroisoandrosterone 3-	C ₂₅ H ₃₆ O ₈	Steroids	464.2447	509.243

Sl/No	RT	Compound	Molecular formula	Class	Molecular mass	<i>m/z</i>
		glucuronide				
19	9.477	Avenanthramide A	C ₁₆ H ₁₃ N O ₅	Carboxylic acid	299.0776	300.085
20	9.876	Eupaformosanin	C ₂₂ H ₂₈ O ₈	Terpene	420.1785	443.1675
21	10.019	Coriandrone E	C ₁₃ H ₁₂ O ₅	Isocoumarins	248.0693	271.0585
22	10.127	Gravacridonetriol	C ₁₉ H ₁₉ N O ₆	Acridines	357.1213	380.1104
23	10.481	(+)-Sophorol	C ₁₆ H ₁₂ O ₆	Isoflavanoid	300.062	301.0693
24	10.623	Isokaempferide	C ₁₆ H ₁₂ O ₆	Flavonoids	300.0663	299.0591
25	10.732	Schizotenuin F	C ₂₈ H ₂₄ O ₁₂	Carboxylic acid	552.1302	299.0529
26	11.044	Hemigossypolone	C ₁₅ H ₁₄ O ₅	Sesquiterpenoid	274.0845	297.0738
27	11.076	3-α(S)-Strictosidine	C ₂₇ H ₃₄ N ₂ O ₉	Alkaloid	530.231	529.2257
28	13.276	Gibberellin A19	C ₂₀ H ₂₆ O ₆	Terpenoid	362.1762	361.1689
29	13.657	Nigakilactone B	C ₂₂ H ₃₂ O ₆	Terpenoid	392.22	415.2091
30	14.041	3-Epinobilin	C ₂₀ H ₂₆ O ₅	Terpenoid	346.1761	369.1653
31	14.171	Cimifugin	C ₁₆ H ₁₈ O ₆	Ketone	306.1106	329.0998
32	11.612	Paradol	C ₁₈ H ₂₈ O ₃	Phenol	292.2042	351.2178
33	15.078	Methyllycaconitine	C ₃₇ H ₅₀ N ₂ O ₁₀	Alkaloid	682.3454	683.3525
34	15.295	Limonoate	C ₂₆ H ₃₄ O ₁₀	Carboxylic acid	506.2183	507.2257
35	15.298	Physalin L	C ₂₈ H ₃₂ O ₁₀	Steroids	528.1999	529.2072
36	13.596	Momilactone B	C ₂₀ H ₂₆ O ₄	Terpenoid	330.1864	329.179
37	14.037	Laserpitin	C ₂₅ H ₃₈ O ₇	Sesquiterpenoid	450.2657	449.2585

Sl/No	RT	Compound	Molecular formula	Class	Molecular mass	<i>m/z</i>
38	16.553	1-(5-Acetyl-2-hydroxyphenyl)- 3-methyl-1-butanone	C ₁₃ H ₁₆ O ₃	Ketone	220.1084	221.116
39	16.714	Palmitic acid	C ₁₆ H ₃₂ O ₃	Fatty acid	272.2359	295.225
40	17.944	Oryzalic acid B	C ₂₀ H ₃₀ O ₅	Diterpene	350.2093	373.1984
41	19.212	Ursolic acid	C ₃₀ H ₄₈ O ₃	Triterpenoid	456.3659	455.3587

Table 25. Phytochemical constituents detected in the methanolic extract from the roots of *V. inermis* plantlets exposed to 200 μ M CuSO₄ on 20d identified through HR LC-MS.

Sl/No	RT	Compound	Molecular formula	Class	Molecular mass	<i>m/z</i>
1	1.315	3,3',5-Trihydroxy-4',7-dimethoxyflavanone	C ₁₇ H ₁₆ O ₇	Flavonoid	332.0897	377.0899
2	1.383	Retronecine	C ₈ H ₁₃ N O ₂	Alkaloid	155.094	156.1013
3	1.389	Chlorogenic acid	C ₁₆ H ₁₈ O ₉	Phenol	354.0942	399.0926
4	6.543	Gibberellin A102	C ₂₀ H ₂₆ O ₇	Terpenoid	378.1678	401.1571
5	6.564	Isoacteoside	C ₂₉ H ₃₆ O ₁₅	Glycosides	624.2114	623.2042
6	6.614	Myricitrin	C ₂₁ H ₂₀ O ₁₂	Flavonoid	464.1	463.0927
7	6.63	Gibberellin A60	C ₁₉ H ₂₄ O ₆	Terpenoid	348.1576	371.1468
8	7.391	Neopetasitenine	C ₂₁ H ₂₉ N O ₈	Alkaloid	423.1887	446.1781
9	8.255	Diosmetin 7-O-beta-D-glucuronopyranoside	C ₂₂ H ₂₀ O ₁₂	Glycosides	476.1001	475.0929
10	8.717	Scutianine F	C ₃₈ H ₄₅ N ₅ O ₅	Alkaloid	651.3461	674.3353
11	8.986	Oroxindin	C ₂₂ H ₂₀ O ₁₁	Carbohydrates	460.1052	459.0979
12	9.027	Loquatoside	C ₂₀ H ₂₂ O ₁₁	Flavonoid	438.1171	461.1056
13	9.081	Dehydroisoandrosterone 3-glucuronide	C ₂₅ H ₃₆ O ₈	Steroids	464.2461	509.2443
14	9.944	Candimine	C ₁₈ H ₁₉ N O ₆	Alkaloid	345.1217	368.1106
15	9.986	Morindone	C ₁₅ H ₁₀ O ₅	Anthraquinone	270.0515	271.0589
16	10.125	Gravacridonetriol	C ₁₉ H ₁₉ N O ₆	Acridines	357.1214	380.1107

Sl/No	RT	Compound	Molecular formula	Class	Molecular mass	<i>m/z</i>
17	10.22	9,10-Dihydroxy-12,13-epoxyoctadecanoate	C ₁₈ H ₃₄ O ₅	Fatty acid	330.244	329.2367
18	10.655	Alangicine	C ₂₈ H ₃₆ N ₂ O ₅	Alkaloid	480.2694	481.2776
19	11.679	Laserpitin	C ₂₅ H ₃₈ O ₇	Sesquiterpenes	450.2663	449.2591
20	13.208	Muscomin	C ₁₈ H ₁₈ O ₇	Flavonoid	346.1057	369.0949
21	14.063	3-Epinobilin	C ₂₀ H ₂₆ O ₅	Terpenoid	346.1761	369.1653
22	14.177	Cimifugin	C ₁₆ H ₁₈ O ₆	Ketone	306.1109	329.1001
23	15.126	Cardiopetalidine	C ₂₁ H ₃₃ N O ₄	Terpenoid	363.2384	364.2456
24	15.534	Momilactone B	C ₂₀ H ₂₆ O ₄	Terpene	330.1869	329.1795
25	15.438	Calycanthine	C ₂₂ H ₂₆ N ₄	Alkaloid	346.2191	391.2172
26	15.577	Prehumulone	C ₂₂ H ₃₂ O ₅	Fatty acid	376.2226	399.2119
27	16.537	1-(5-Acetyl-2-hydroxyphenyl)-3-methyl-1-butanone	C ₁₃ H ₁₆ O ₃	Ketone	220.1086	221.1161
28	16.662	Palmitic acid	C ₁₆ H ₃₂ O ₃	Fatty acid	272.236	295.225
29	17.837	Oryzalic acid B	C ₂₀ H ₃₀ O ₅	Diterpene	350.2097	373.1981
30	17.872	(S)-Neomammein	C ₂₂ H ₂₈ O ₅	Coumarins	372.1909	395.18
31	19.178	Ursolic acid	C ₃₀ H ₄₈ O ₃	Triterpenoid	456.3664	455.3592

1.418 to 19.211. The first resolved compound is an alkaloid called retronecine and the last resolved compound was ursolic acid, which is a triterpenoid compound. The liquid chromatogram of both positive and negative ionization modes of the extract is given in Figure 46. In the control roots, alkaloids, terpenoids and flavonoids are the major class of compounds. Glycoside compounds were also detected in the control roots. When compared to the control root extract, number of phytochemicals were very high in both NaCl and CuSO₄ treated roots. Total 41 compounds (Fig. 47 and Table 24) were resolved in the NaCl treated root extract whereas 31 different phytochemicals (Fig. 48 and Table 25) were observed in CuSO₄ treated roots. In the NaCl treated roots, retronecine and ursolic acid was the first and last resolved phytochemicals and it was similar to that of control root extract. And the retention time ranged from 1.397 to 19.212. Besides the alkaloids, terpenoids, flavonoids and glycosides, phenolic compounds were also detected in the NaCl treated root extract. But phenolic compounds were not observed in the control root extract. Gallic acid, caffeic acid, ferulic acid, oolonghomobisflavan A and paradol are the phenolic compounds found in NaCl treated roots. Chlorogenic acid was the only phenolic compound found in CuSO₄ treated roots. In the case of CuSO₄ treated roots the first resolved compound was 3,3',5-Trihydroxy-4',7- dimethoxyflavanone with a retention time of 1.315. Here also the last resolved compound was ursolic acid with 19.178 retention time. When compared to the control and NaCl treated root extract, anthraquinone was only found in CuSO₄ treated roots and that was morindone. Various coumarin compounds were also identified in all the three extracts. They are feruloylputrescine, 5,7-dihydroxychromone, coriandrone E and (S)-neomammein. Moreover, all the

three extracts contained a significant number of alkaloids, terpenoid and flavonoid phytochemicals.

4.16 Gene expression studies

4.16.1 Phytochelatase

RT-qPCR analysis was used to assess the *pcs* transcript levels in the leaves and roots of *V. inermis*. The enhanced expression of *pcs* was found in the leaves as compared with the roots. The leaves of the plantlets treated with 400 mM NaCl exhibited 3-fold increase in the *pcs* gene expression level as compared with the leaves of control plantlets. At the same time the roots of plantlets treated with 400 mM NaCl exhibited 4-fold increase in the *pcs* gene expression level as compared with the roots of control plantlets (Fig. 49).

The 200 μ M CuSO₄ treatment in the *V. inermis* also exhibited similar trend of enhancement. The leaves of the plantlets treated with 200 μ M CuSO₄ exhibited an enhancement of 7-fold as compared with the leaves of control plantlets. In the case of roots, the enhancement in the *pcs* gene expression was 2-fold higher as compared with the roots of control plantlets (Fig. 49).

4.16.2 Phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase gene expression level was higher in the roots as compared with the leaves. The leaves of the plantlets exposed to 400 mM NaCl showed 1-fold increase in the *pal* expression as compared with the leaves of control plantlets. But in the case of roots, exposure to 400 mM NaCl

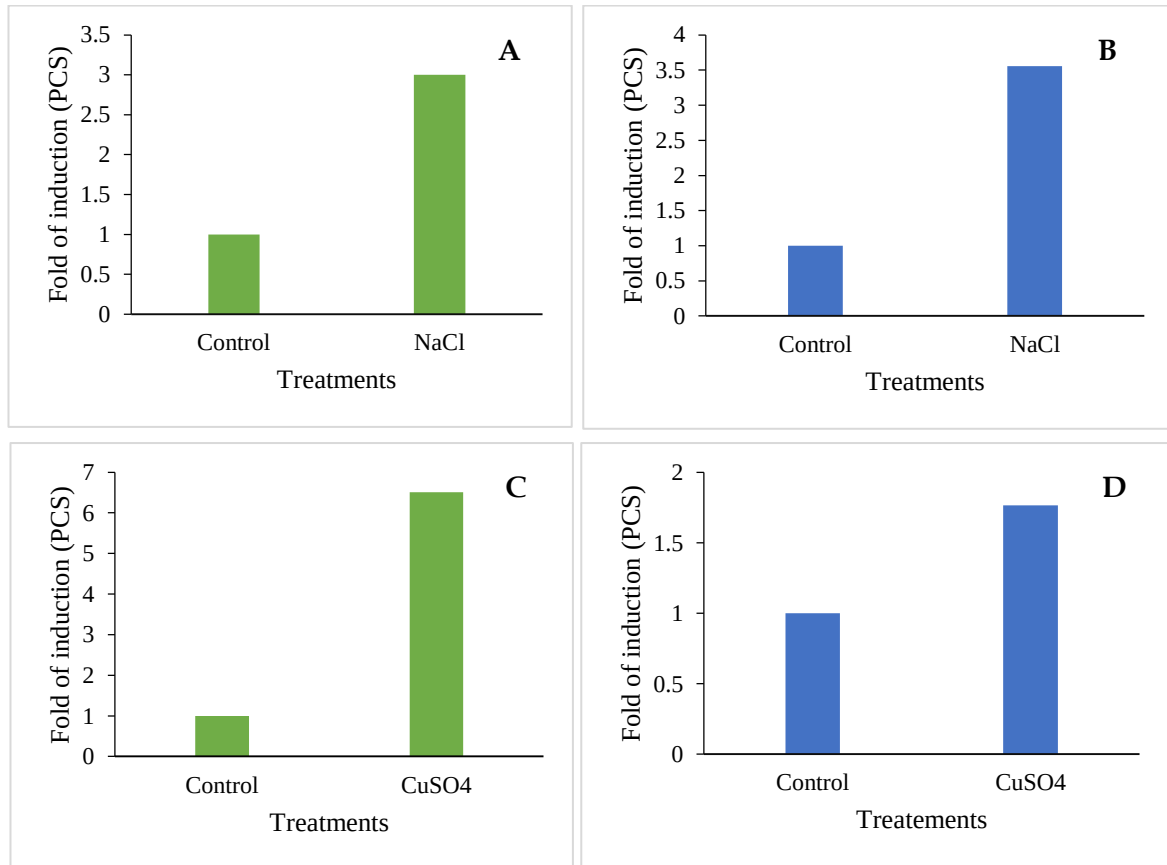


Figure 49. Gene expression of Phytochelatin synthase (PCS) in the leaves (A and C) and roots (B and D) of *V. inermis* plantlets treated with 400 mM NaCl and 200 μ M CuSO₄. The gene expression in terms of fold of induction with respect to control plantlets.

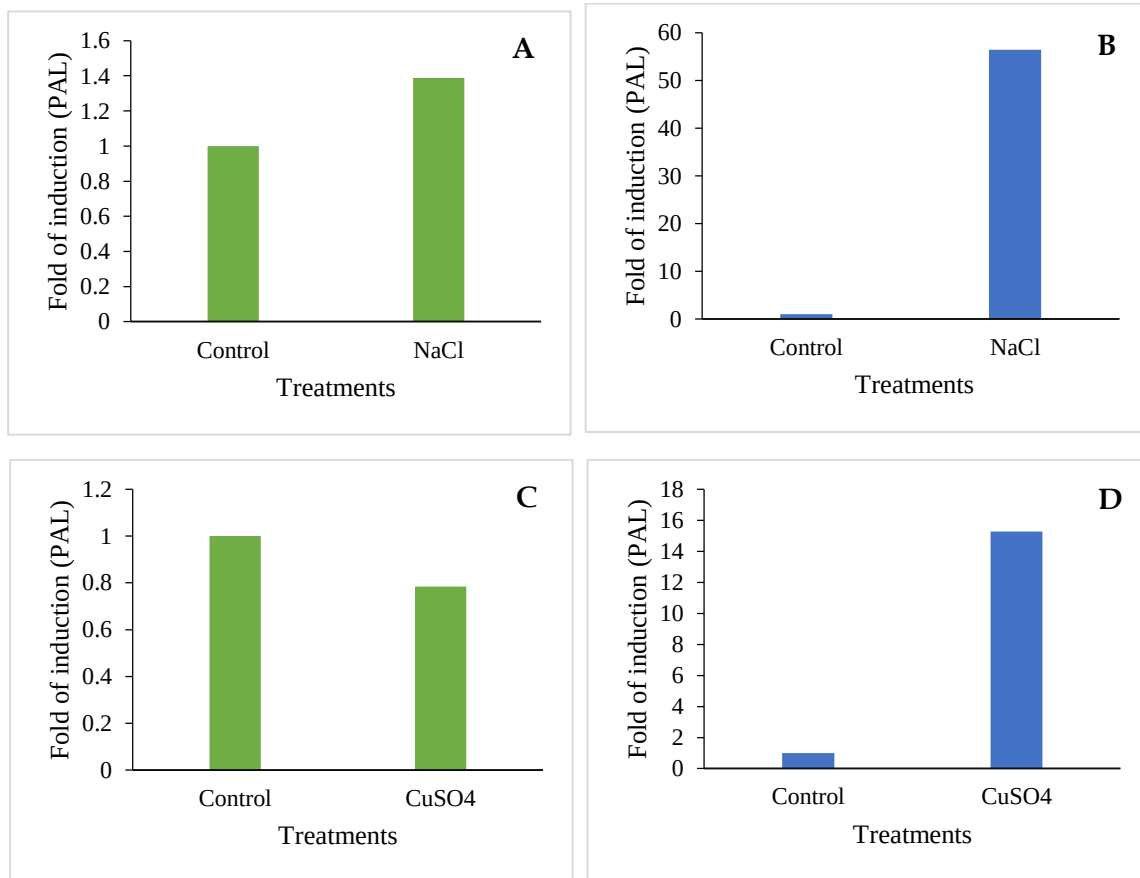


Figure 50. Gene expression of Phenylalanine ammonia-lyase (PAL) in the leaves (A and C) and roots (B and D) of *V. inermis* plantlets treated with 400 mM NaCl and 200 μ M CuSO₄. The gene expression in terms of fold of induction with respect to control plantlets.

resulted in 56-fold enhancement in *pal* expression as compared with the roots of control plantlets. The exposure of *V. inermis* to 200 μ M CuSO₄ resulted in higher *pal* expression in roots as compared with the leaves. The roots showed a higher level of *pal* gene expression and it was 15-fold higher as compared with the control plantlets (Fig. 50).

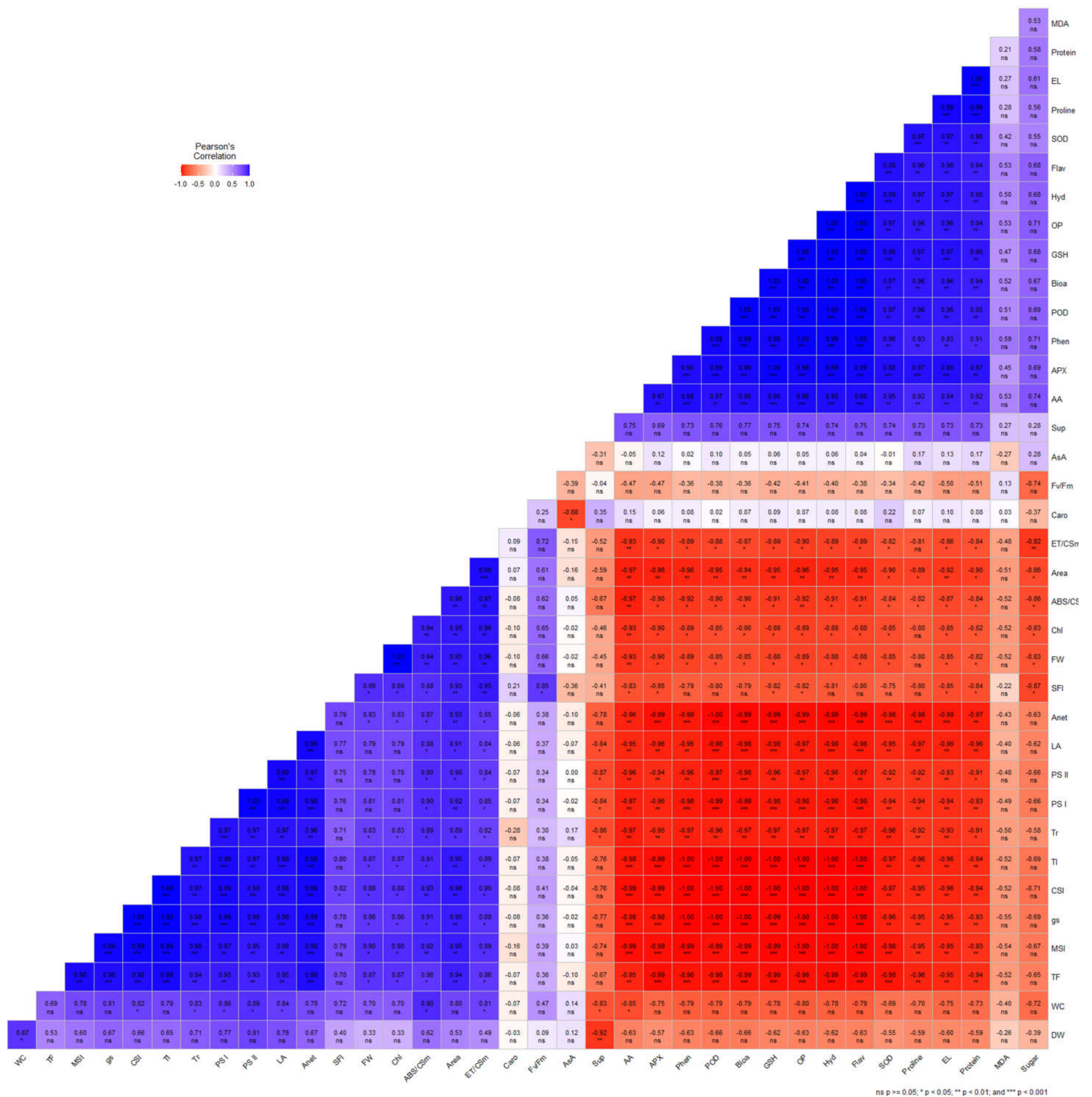


Figure 51. The correlation matrix heatmap of leaves of the plantlets treated with NaCl showing the values of the Pearson correlation coefficient between parameters. The positive values are represented in blue and negative in red. It ranges from -1 to 1, whereby -1 means a perfect negative linear relationship between variables, +1 indicates a perfect positive linear relationship between variables and 0 indicates that there is no relationship between studied variables.

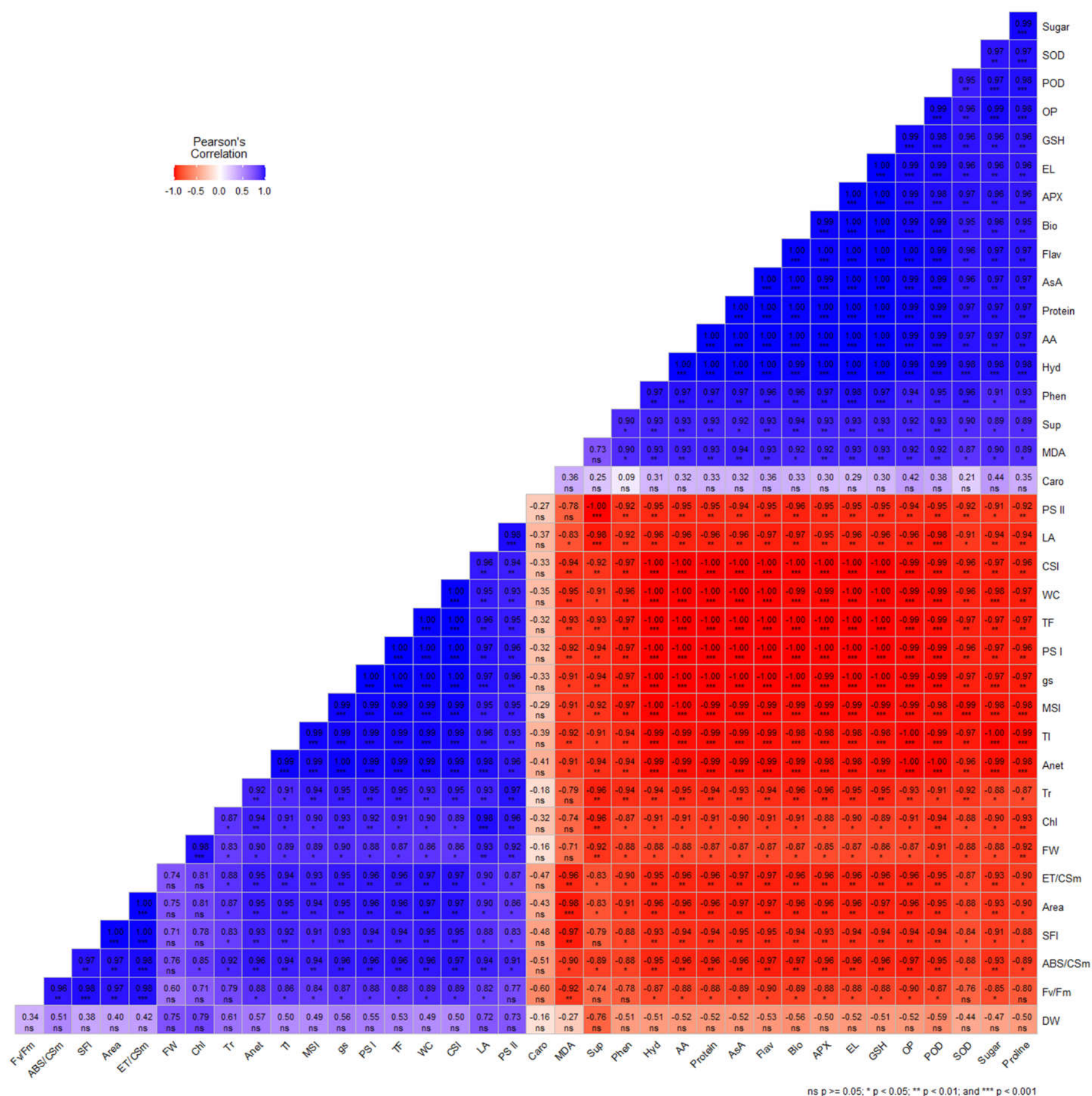


Figure 52. The correlation matrix heatmap of leaves of the plantlets treated with CuSO₄ showing the values of the Pearson correlation coefficient between parameters. The positive values are represented in blue and negative in red. It ranges from -1 to 1, whereby -1 means a perfect negative linear relationship between variables, +1 indicates a perfect positive linear relationship between variables and 0 indicates that there is no relationship between studied variables.

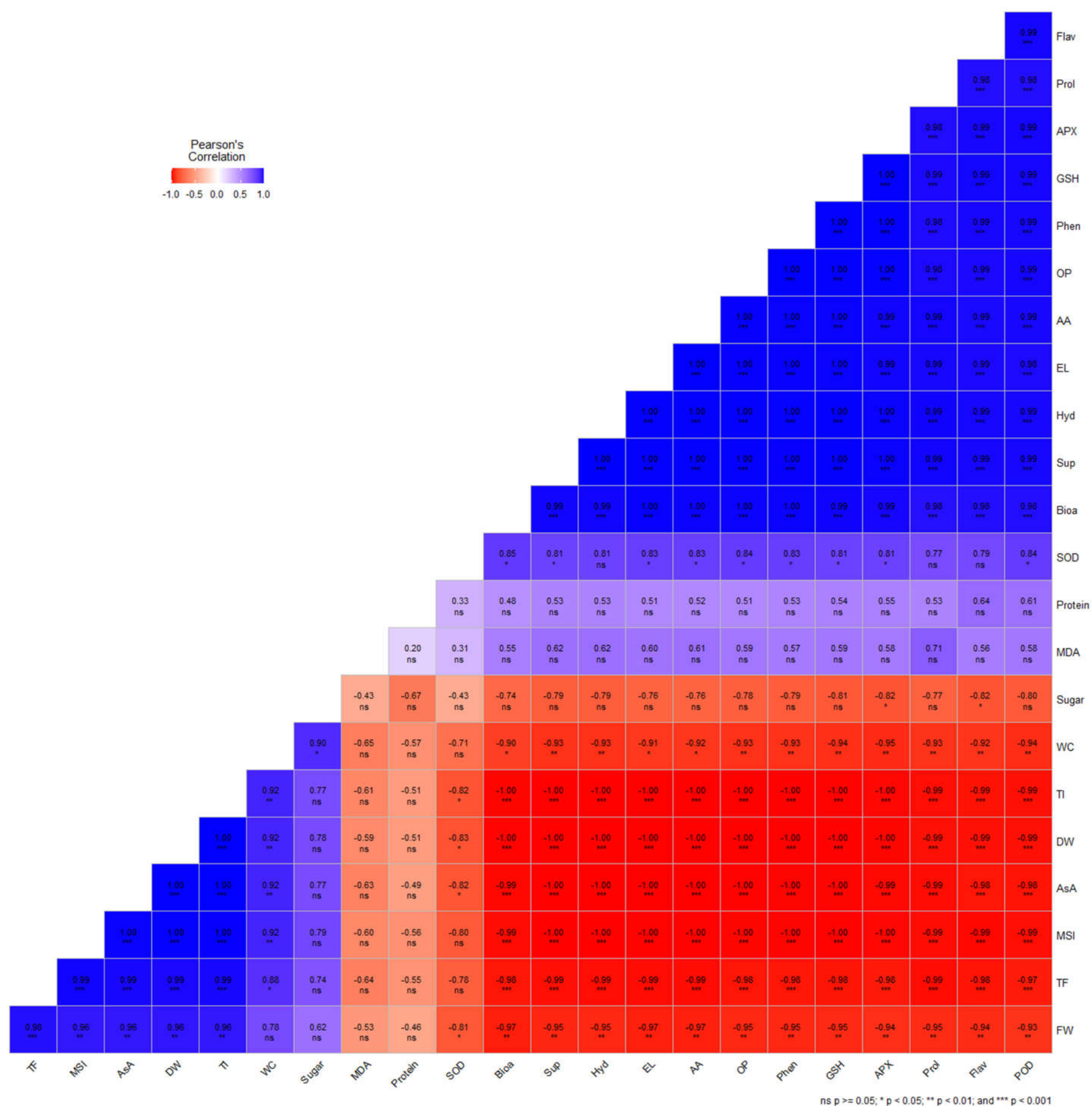


Figure 53. The correlation matrix heatmap of roots of the plantlets treated with NaCl showing the values of the Pearson correlation coefficient between parameters. The positive values are represented in blue and negative in red. It ranges from -1 to 1, whereby -1 means a perfect negative linear relationship between variables, +1 indicates a perfect positive linear relationship between variables and 0 indicates that there is no relationship between studied variables.

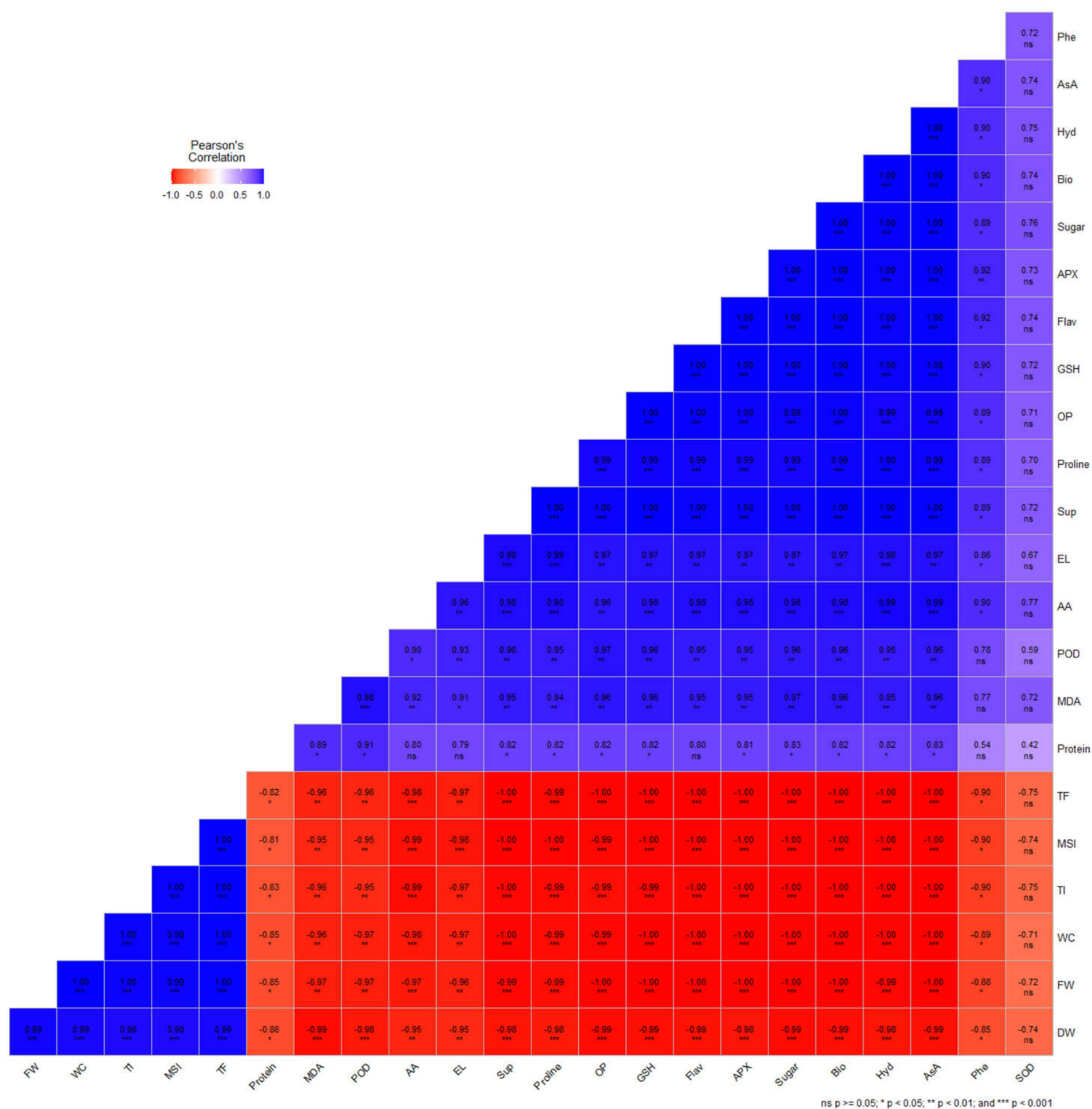


Figure 54. The correlation matrix heatmap of roots of the plantlets treated with CuSO₄ showing the values of the Pearson correlation coefficient between parameters. The positive values are represented in blue and negative in red. It ranges from -1 to 1, whereby -1 means a perfect negative linear relationship between variables, +1 indicates a perfect positive linear relationship between variables and 0 indicates that there is no relationship between studied variables.

5. DISCUSSION

The lack of precipitation, and irrigation with saline water contribute towards soil salinization. Among the different abiotic factors, soil salinity is a major issue affecting plant growth and metabolism, thus agriculture and food security. Unjudicious agricultural practices including over use of chemical pesticides affect the soil fertility and increase the accumulation of heavy metals like Cu. NaCl and Cu are necessary for plants to sustain normal functions, but higher concentration of these two in the soil can inhibit plant metabolic processes. Salinity induces osmotic stress, which induces physiological dryness in the plant, resulting in stunted plant growth. Similar way, copper also affects negatively if the concentration of the same is greater than the optimal. The higher concentration of copper affects the key metabolic processes including photosynthesis.

5.1 Plant growth

The increased salinity level in the growing habitats is generally a serious problem for all plants. The supra/sub-optimal concentration of salinity affects plant growth negatively. On exposure of plants to salinity stress, plant growth is limited due to a reduced rate of photosynthesis, and the energy is maximally utilized for survival strategies. In this study, the fresh and dry weight of the leaves and roots of NaCl-treated plantlets declined proportionally with increasing NaCl concentration, except for those treated with 100 mM NaCl. The promotion of plantlet growth by a modest concentration of NaCl (100 mM) compared to control plantlets confirms the halophytic nature of *V. inermis*.

The growth of plantlets under CuSO₄ treatment experienced the same condition with higher fresh and dry weight reduction only in higher

concentration of CuSO_4 . The essentiality of the Cu for plant growth was explained by many authors and Cu was categorised as an important micronutrient. The optimal level of Cu in the growth medium promote plant growth due to its essentiality. And this was observed in the present study wherein, plantlets which were grown in the lower CuSO_4 concentration (100 μM) did not affect the fresh weight except on 20d, where there was slight reduction (5%) as compared to the control plantlets. But at the same time the higher CuSO_4 concentration (200 μM) reduced the fresh weight by 63% as compared control plantlets. Mateos-Naranjo et al. (2013) reported that, higher Cu has growth retarding properties and cause yellowing of the leaves due to degraded or lower synthesis of plant pigments, decreased root growth and anomalous branching. These responses ultimately results in the decreased plant growth, which was reflected as reduced fresh and dry weights. Sruthi and Puthur (2019) reported the reduction in biomass in terms of dry weight in the halophyte *Bruguiera cylindrica* under Cu stress. Among the different concentrations of Cu treatment, 0.25 mM caused highest reduction in dry weight and the effects were more reflected in roots than leaves. Similar observations were also found in *V. inermis* in response to CuSO_4 . There was a reduction of 58 and 84% dry weight in shoot and roots of *Salix* species on exposure to 0.25 mM CuSO_4 (Kuzovkina et al., 2004).

The enhancement in the leaf area of *V. inermis* treated with lower concentration of NaCl (100 mM) clearly indicates the beneficial effect lower NaCl concentration on plant growth. But at the same time, plants grown in other higher concentrations of NaCl (200, 300 and 400 mM) had leaves with reduced leaf area in comparison with control. A linear correlation between salinity concentration and leaf area was observed in the case of this halophyte. The reduction in the expansion of leaves was due to the reduced cell division, ultimately causing the reduction in total dry mass (Lovelock and Ball, 2002; Alharby et al., 2014). Abiotic stresses cause a reduction in leaf

area and this was reported by many authors. The reduced leaf area in the plantlets exposed to NaCl significantly contribute to its water use efficiency by reducing the transpiration rate. The absorbed water is effectively conserved by reducing the leaf area (Oraby and Ahmad, 2012; Schnaubelt et al., 2013; Tari et al., 2013; Balfagón et al., 2020). The reduced leaf area observed in *V. inermis* in response to CuSO₄ treated plantlets could also be due to the suppression of the cell division and elongation, due to an irreversible inhibition exerted by higher concentration of Cu on the proton pump responsible for the cell expansion process (Jiang et al., 2001; Qin et al., 2015).

One of the most frequently employed criteria to evaluate NaCl and CuSO₄ tolerance in plants is TI%. When selecting plants for use in phytoremediation applications, it is very important to evaluate the metal tolerance of the plants. The TI of *V. inermis* was increased when exposed to 100 and 200 mM NaCl, but it declined when subjected to higher NaCl concentrations (300 and 400 mM). The enhancement in growth under low concentrations of NaCl was evident from the parameter TI too. According to Ma et al. (2013), the ideal salinity for maximal growth of halophytes is typically between 100 and 200 mM NaCl, and its growth is greatly inhibited if the salinity of the soil is beyond this range. The results of this study corroborate the observation of Ma et al. (2013). Most of the earlier investigations have indicated that growth rate and dry matter accumulation increase with salt concentration in the range 100–200 mM. The maximal tolerance concentration may vary with respect to each species (Bueno and Cordovilla, 2021; Kumar et al., 2021b). The limited growth under NaCl stress was due to the utilization of the energy for energizing tolerance mechanisms, and it was reflected in reduced fresh and dry weight. The presence of high concentration of Na⁺ and Cl⁻ in the growing habitat and excessive uptake of these ions into the plant body alter the ion homeostasis,

thereby altering the optimal functioning of physiological processes. Moreover, the salinity stress in plants creates a physiological drought condition, leading to stunted growth (Ahammed et al., 2020; Sheldon et al., 2020). The decreased biomass in the salt stress condition was due to the lower content of photosynthetic pigments and the associated photosynthetic functions, resulting from an increase in the chlorophyll degrading enzyme, chlorophyllase (Palliyath and Puthur, 2018). The reduction in the chlorophyll content and, thereby, decrease in plant biomass was reported in the plants like *Aegiceras corniculatum*, *Ceriops roxburghiana*, and *Atriplex halimus* under high salinity, however these plants showed better performance in lower saline concentrations (Natarajan and Chellappan, 2004; Parida et al., 2004a; Palliyath and Puthur, 2018).

The plantlets on exposure to 200 μM CuSO_4 , TI% of leaves were reduced to 50% as compared to the control plants. Similar trend of reduction was also observed in the case of roots. The TI% was 80 and 54% in roots of plantlets treated with 100 and 200 μM CuSO_4 respectively as compared with the control plantlets. The efficacy of the plantlets to thrive under metal stress was effectively measured by TI%. The reduction in biomass and TI% was reported in the halophytes such as *A. ilicifolius* and *B. cylindrica* (Shackira and Puthur, 2017; Sruthi and Puthur, 2019). The effect of CuSO_4 on leaves and roots of *V. inermis* was reflected in TI%. The reduced biomass of leaves and roots adversely affect the TI%. The stress induced root growth inhibition allows the plant to minimize the exposure surface of the roots to the metal and thereby make them more tolerant. The plants growing under higher concentrations of NaCl and CuSO_4 had lower uptake of minerals and reduced rate of water intake. These abnormal conditions severely affect the plant growth especially the cell division of root and cell cycle events (Muhammad et al., 2015).

The tissue water content indicates the ability of the plants to retain water under stressful conditions by providing an estimate of the mass of water in the tissues in proportion to their maximal water holding capacity (Chaparzadeh et al., 2003). The tissue water content of the leaves and roots of plantlets was reduced compared to the respective control plantlets. The maximum reduction was found in plantlets grown in higher NaCl concentrations (400 mM). Similar results were observed in the halophytic plant, *Bruguiera cylindrica*, when subjected to 600 mM NaCl (Palliyath and Puthur, 2018). The hyperabsorption of Na^+ and Cl^- lead to the reduction of cellular water potential in NaCl stressed plant. It acts as an adaptation in halophytes, which helps them to maintain osmoticum and ensure continuous water absorption through roots. Maintenance of appropriate water potential gradient allows the entry of water into the plant, which aids in maintaining cell turgor and enlargement, ultimately promoting plant growth under NaCl stress (Munns and Tester, 2008; Hasanuzzaman et al., 2014a). Like NaCl, higher concentration of CuSO_4 negatively affect the tissue water content. According to Saied et al. (2005) and Kaya et al. (2007), the metal uptake in plants negatively affect the tissue water status due to impairment in the absorption of water. The heavy metal induced impairment in plant-water relationship is due to stress induced down regulation of root hair formation and it results in the reduction of surface absorption, bio-membrane permeability and xylem differentiation and these features directly affect the tissue water status (Pál et al., 2006; Hu et al., 2020; Zhang et al., 2023a). The strong negative correlation of tissue water content and Na/Cu accumulation in leaves (-0.993 and $p \leq 0.01$ in CuSO_4 treatment) and roots (-0.896 and $p \leq 0.05$ in NaCl treatment and -0.998 and $p \leq 0.01$ in CuSO_4 treatment) clearly indicates that tissue water imbalance was due to Na/Cu accumulation in *V. inermis*.

5.2 Stress intensity factors

Biotic or abiotic stressors, alter plant's normal physiology and metabolism. Among them one of the major metabolic changes that take place in the plant cell is the increase in the level of reactive oxygen species (ROS) (Allen, 1995). The key ROS producers within plant cells include the mitochondria and chloroplast. Under the stress condition, superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\bullet}$), and other activated oxygen species are produced during aerobic metabolism as a result of some electrons that leak out during normal electron transport in the mitochondria and chloroplasts (Asada, 1999; Anjum et al., 2022). The first stage in the synthesis of ROS is the reduction of molecular oxygen by a single electron, which results in the production of superoxide radical ($O_2^{\bullet-}$) (Foyer and Hanke, 2022).

Salinity and heavy metal stress impart oxidative stress by producing ROS (Nagajyoti et al., 2010). Heavy metals react with different proteins in the chloroplast and mitochondria causing obstruction in the smooth flow of electron and thus over reduction of the electron transport chain (ETC) occurs, leading to the generation of ROS, causing metabolic impairment (Lokhande and Suprasana, 2012). The over accumulation of heavy metals like Cu, Cr, Fe and Co in plants leads to ROS formation through Fenton-type reaction and other metals (called non redox metals) also produce ROS by different mechanisms like NADPH oxidase-dependent H_2O_2 accumulation and $O_2^{\bullet-}$ formation in mitochondria (Garnier et al., 2006). ROS reacts with lipids, proteins, pigments and nucleic acids and cause lipid peroxidation, membrane damage, and inactivation of enzymes, thus resulting in toxic effects on plants. The inactivation and denaturation of proteins occurring during heavy metal stress is due to the affinity of metals towards the S and N atoms of the amino acids present in the proteins. These heavy metals also

interfere with the sulphhydryl groups present in the enzymes and structural proteins and it also displaces the cations in the active binding site of the enzymes (Sharma and Dietz, 2009).

The rate of synthesis of ROS and ROS induced damages primarily indicate the stress intensity encountered by plants under abiotic factors. Shahid et al. (2014) reported that the augmented production of ROS and resulting oxidative stress is the first line of toxicity towards heavy metals stress. The oxidative stress affects the normal metabolism of plants and cause distortion to macromolecules (Ajayan and Selvaraju, 2012). The exposure of *V. inermis* to NaCl treatments cause oxidative stress by the upregulation of the ROS such as H_2O_2 and $\text{O}_2^{\bullet-}$ due to the damage of the photosystems. The production of H_2O_2 and $\text{O}_2^{\bullet-}$ was higher in higher NaCl concentrations than the plantlets grown in the lower NaCl concentrations. In the case of leaves, the leakage of electrons leading to $\text{O}_2^{\bullet-}$ accumulation occur during the transfer of electrons between Q_A and Q_B and in Fe-S clusters of PSI (Sharma et al., 2012; Kostecka-Gugała and Latowski, 2018). The severe damages of the PSI and PSII including the aberrations on electron transport system, plastoquinone pool etc. in the plantlets of *V. inermis* clearly defines the augmented rate of $\text{O}_2^{\bullet-}$ radical production.

In NaCl treated plantlets, the gas exchange was limited due to several reasons. The decreased gas exchange resulted in photorespiration where the ribulose-1, 5-biphosphate carboxylase/oxygenase (Rubisco) binds with oxygen instead of CO_2 . As a result, the production of phosphoglycolate was increased due to the photorespiration. During this process, H_2O_2 is produced in peroxisomes by glycolate oxidase (Bauwe et al., 2012; Hameed et al., 2021). This could be one of the major routes of H_2O_2 increase in *V. inermis* in response to higher NaCl concentration. Besides this, the mitochondrial electron transport under NaCl or CuSO_4 stress also lead to the production of

ROS. Superoxide levels rise as a result of the over-reduction of the electron transport chain in mitochondria. The higher synthesis of H_2O_2 in response to NaCl was reported in the halophyte *Thellungiella salsuginea* (Wiciarz et al., 2015). Similarly, higher production of ROS and subsequent decrease in photosynthesis was reported in the halophyte *Avicennia marina* grown under 500 mM NaCl (Jithesh et al., 2006). The augmentation of H_2O_2 content as well as the antioxidant enzymes such as ascorbate peroxidase (APX), guaiacol peroxidase (POD), glutathione reductase (GR), and superoxide dismutase (SOD) were reported in *Bruguiera parviflora* under salt stress (Parida et al., 2004b). The production of superoxide radicals and thereby subsequent activation of SOD increase the amount of H_2O_2 , which was clearly evident from this study.

Like NaCl treatment, the plantlets treated with CuSO_4 recorded significant increase of $\text{O}_2^{\cdot -}$ content in leaves and roots. In leaves, $\text{O}_2^{\cdot -}$ content was increased to 2.6 and 4-fold in plantlets treated with 100 and 200 μM CuSO_4 respectively as compared with the control plantlets. Similar kind of increase was found in roots and it was more prominent than leaves. The increase was 6 and 15-fold in roots treated with 100 and 200 μM CuSO_4 respectively as compared with the control plantlets. The excess of heavy metals like Cu, Cr, Fe, V and Co in the growth medium have the capacity to produce ROS via Fenton-type reactions. The individual and combined stress of CuSO_4 and NaCl in the halophyte *B. cylindrica* caused an increase of ROS (Sruthi and Puthur, 2020). This author found that, under combined stress conditions, even with modest CuSO_4 concentration, the NaCl tolerance potential of *B. cylindrica* was reduced. Previous studies have shown that Cu and other heavy metals like Cr, Zn, and Ni generate ROS such as $\text{O}_2^{\cdot -}$ and H_2O_2 , which in turn cause oxidative damage and cell death in plants. The unregulated electron flow due to the augmented oxidation of metabolites in cell organelles including chloroplast, peroxisomes and mitochondria in

heavy metal treated plants causes a rapid increase in ROS production (Rastgoo and Alemzadeh, 2011). The damages to the D1 and D2 proteins due to the heavy metal induced substitution of Ca^{2+} and Mg^{2+} results in the impairment of PSII and thereby water oxidizing complex and it results in the uncoupling of electron transport chain in the chloroplast (Solanki and Dhankhar, 2011). The enhancement in the superoxide radicals and hydrogen peroxide upto six-fold and three-fold in a halophyte *Kosteletzkya virginica* exposed to Cd (5 μM) and similar increase of hydrogen peroxide content in response to heavy metals like Cd^{2+} , Ni^{2+} and As^{3+} in *Salicornia brachiata* was reported earlier (Sharma et al., 2010a,b; Han et al., 2013).

During the onset of severe stress, the membrane fluidity was totally decreased, which augmented the leakage and caused damages to membrane bound and peripheral proteins. This was due to the peroxidation of polyunsaturated fatty acids, the major membrane lipids of plant cell membranes, which are predominantly vulnerable to ROS attack (Halliwell, 2006; Ahmad et al., 2010). The ROS generation in response to NaCl and CuSO_4 resulted in the peroxidation of membranes. Lipid peroxidation, which is caused by a high concentration of ROS in the cellular environment, harms the structure of cells and biomolecules by damaging DNA, proteins, and lipids in cell membranes. The ROS induced damages through lipid peroxidation was assessed by analysing MDA content. It is a decomposition product of polyunsaturated fatty acids. The ROS has the potential to remove the hydrogen from the unsaturated fatty acids, leading to the formation of lipid radicals. By this process, the lipid bilayer membrane of the plant cells was totally distorted. The polymerisation and dimerization of membrane proteins due to lipid peroxidation severely damages the plant cells and it affect the normal functioning of plants (Demidchik, 2014; Hasanuzzaman et al., 2021). The result of this study also corroborates with the increasing trend of MDA content observed in response to increasing NaCl and CuSO_4

concentration in the medium. The plantlets exposed to lower concentration of NaCl and CuSO₄ had lesser amount of MDA and it clearly indicates the lower level of lipid peroxidation in *V. inermis*.

Similar trend of increase was also observed in the roots of plantlets treated with NaCl and the highest MDA content was found in the root tissues of the plantlets treated with 400 mM NaCl (4.3 fold) as compared with the control plantlets. As roots are the first and over exposed plant part than the leaves, the MDA content in the roots are found to be much higher than the leaves. This can severely affect the root growth than leaves. The high salinity and heavy metal concentration in the growth medium and the accumulated sodium ions and metals in the plant parts reduce the amount of lipid biosynthesis and thereby reduce the lipid content. Moreover, abiotic stress has also a stimulative response on the enzymes for lipolysis and lipid peroxidation (Gogna and Bhatla, 2020). The leakage of the electrolytes due to the higher ROS accumulation in NaCl and CuSO₄ treated plants severely affects the stability of the membranes (Li et al., 2008; Yasar et al., 2010; Guo et al., 2019). The higher content of MDA not only hinders the bio-membrane permeability, but also cause leakage of electrolytes and inactivation of membrane receptors and enzymes.

At severe cases, the oxidation of DNA, RNA and proteins are also generally observed. The chloroplast and mitochondrial DNA is more prone to oxidation than the DNA present in the nucleus, because of its proximity to the production sites of ROS (Das and Roychoudhury, 2014; Anjum et al., 2015). This was clearly evident from the studies with *V. inermis* plantlets subjected to higher NaCl concentrations, which negatively affects the stability of cell membranes. A significant reduction of MSI was recorded in plantlets exposed to 400 mM NaCl concentration on 20d with respect to the control plants. In this study, the MSI of plantlets treated with lower

concentration of NaCl was better than that of the control indicating the essentiality of minimum level of NaCl in the growth medium in which mangrove associates are grown. Similar trend of results was also observed in the plantlets treated with CuSO₄. Mild concentration (100 µM) did not alter the MSI and it was maintained as like that of control plantlets. The higher CuSO₄ (200 µM) treatment resulted in significant reduction of MSI than the control leaves.

5.3 Photosynthetic efficiency

5.3.1 Photosynthetic pigments and chlorophyll stability index

Stress caused by saline environments in plants results in nutrition depletion and enhance various plant senescence mechanisms which can lead to decreased chlorophyll content of leaves (Munns and Tester, 2008; Petretto et al., 2019). In the present study, the total chlorophyll content in the leaves of the plantlets decreased with increasing concentrations of NaCl, except the plantlets subjected to 100 mM NaCl. Salt tolerant species like *Thellungiella halophila* have been found to thrive in NaCl stressed conditions (upto 0-500 mM) without a significant decrease in chlorophyll content (Stepein and Johnson, 2008). In some cases, even an increase in chlorophyll content have been reported under low concentrations of NaCl, similar to that observed in the chlorophyll content of *V. inermis*. The plantlets grown under 100 mM NaCl exhibits higher chlorophyll content than control plantlets. The treatment with 50 mM NaCl in the halophytic plant, *Tetragonia decumbens* resulted in an increased chlorophyll content than the control. However, increased salinity decreased the chlorophyll content in many plants (Sogoni et al., 2021). Takagi and Yamada (2013), Koyro (2006) and Megdiche et al. (2007) observed a similar decrease in chlorophyll content in the halophyte *Kochia scoparia*, *Plantago coronopus* and *Cakile maritima* plants under increasing concentrations of NaCl. This could be due to the enhanced

activity of the pigment degrading enzyme chlorophyllase (Iyengar and Reddy, 1996). The bioaccumulation of Na ions in the plant parts cause the degradation or lower the synthesis of chlorophyll pigment, reflected in the lower chlorophyll content as evident from this study. The negative correlation between bioaccumulation of Na with chlorophyll pigment ($r = -0.86$ and $p \leq 0.05$) validates the same. The plantlet productivity under abiotic stress condition directly depend upon the chlorophyll pigment, because it is the prime factor for photosynthesis. Upon exposure to higher NaCl concentration, the TI was also reduced in *V. inermis*. The tolerance index directly indicates the plantlets tolerance efficiency under stress. In this study the TI of the plantlet grown in 400 mM NaCl was 41% than the control plantlets. The reduction in TI with increment in the concentration of NaCl was positively correlated ($r = 0.87$ and $p \leq 0.05$).

The present study found that lower concentration of CuSO_4 positively influenced and enhanced the total chlorophyll content over control. This indicates that the plant is tolerant to lower concentrations of Cu and also it has specific role in plant growth and development. The optimal concentration of Cu in the growth habitat promote plant growth and play vital roles in in photosynthetic and mitochondrial electron transport, cell wall metabolism and hormone signalling (Shabbir et al., 2020; Wairich et al., 2022). Similar to our study, it was earlier found that 50-200 ppm of CuSO_4 maintained the chlorophyll content in the halophyte *Mesembryanthemum crystallinum* as that of the control plant (Thomas et al., 1998). However, increased concentrations of Cu have been found to reduce the chlorophyll content of *V. inermis*. Similar to our study, *Atriplex halimus* exhibited a reduction in chlorophyll content under higher Cu stress (Brahim and Mohamed, 2011). According to John et al. (2009), decreased chlorophyll content could be due to the degradation of enzymes involved in the biosynthesis of chlorophyll. The chlorophyll molecules may also be

destroyed by the replacement of Mg in the chlorophyll by the heavy metals like Zn, Cu or Cd (Prasad and Strzalka, 1999). The higher Cu in the growing habitat compete with Mg and Fe leading to its decreased uptake. The biosynthesis of chlorophyll is strongly impacted by the increased Cu and decreased Mg and Fe contents. Moreover, the intermediate product in the formation of chlorophyll, protochlorophyllide, is inhibited by higher Cu concentrations (Vijayarengan and Jose, 2014). The higher Cu in plant body potentially bind with the -SH groups of the aminolevulinic acids (ALA) dehydratase and protochlorophyllide reductase and thereby reduces its enzymatic capacity and inhibits chlorophyll biosynthesis (Myśliwa-Kurczel et al., 2004). The reduction in chlorophyll content of *V. inermis* was majorly due to the accumulation of Cu in the different plant parts. Chlorophyll content was decreased due to Cu accumulation in the plant components, as demonstrated by the negative correlation between chlorophyll content and Cu bioaccumulation in leaves ($r = -0.91$ and $p \leq 0.05$).

Carotenoids are important components that protect the photosynthetic machinery of plants (Ghanem et al., 2021). The carotenoid content in the leaves of *V. inermis* increased in response to both NaCl and CuSO₄ treatments. Similar results were observed by Ghanem et al. (2021) wherein, they found that as the concentration of NaCl increased in the medium, an elevation in the carotenoid content in the leaves of *Arthrocnemum macrostachyum* and *Sarcocornia fruticosa* occurred. They attributed this increase as a strategy to protect the chlorophyll and the photosynthetic machinery in the leaves. *Sesuvium* plants exhibited an increase in carotenoid content when grown under 400 mM NaCl (Ramani et al., 2006). The enhanced carotenoid content offers increased protection to the photosynthetic machinery in plants subjected to salt stress (Muchate et al., 2016). The role of carotenoids in reducing the triplet state of chlorophyll and thereby eliminating the chance of singlet oxygen formation is a major

strategy of imparting protection to the photosynthetic machinery (Ramani et al., 2006). Heavy metal stress has been found to decrease the carotenoid content of halophytes like *Atriplex halimus* (Mesnoua et al., 2016). However, in the present study, CuSO_4 increased the carotenoid content of *V. inermis*. This could be because carotenoid production may be dependent on the type of heavy metals. Some heavy metals may trigger carotenoid accumulation while others may reduce it (Fargasova, 1998; Singh and Tewari, 2003). So, it is well clear that enhanced carotenoid content is an adaptive strategy to protect the plants from stress induced injury.

CSI has been reported to be an important marker of stress tolerance capability of plants (Mohan et al., 2000). Gadi and Goswami (2016), Agarwal and Shaheen (2007), Gadi et al. (2016) and Ahmad et al. (2008) have reported decreased CSI in *Lasiurus scindicus*, *Momordica charantia*, *Vigna radiata* and *Pisum sativum* respectively, in response to increasing concentrations of NaCl in the growth medium. Premature senescence of leaves under salt stress is an after effect of decreasing CSI in plants (Dutta and Bera, 2014). As revealed from this study, CSI of *V. inermis* decreased under increasing concentrations of NaCl. However, 100 mM NaCl resulted in an increase in CSI which points out that this concentration is not a stress on *V. inermis*. Higher CSI values in plants under stressed conditions depicts that the plants are resistant to that particular stress (Mohan et al., 2000). *V. inermis* plants treated with CuSO_4 also exhibited a similar trend as that of NaCl stress. 100 μM CuSO_4 resulted in a slight enhancement in CSI. However higher concentrations reduced the CSI of *V. inermis* plants. This indicates that the plants are tolerant to lower concentrations but susceptible to higher concentrations of CuSO_4 . Cu stress was found to reduce the CSI of cotyledonary leaves of *Ricinus communis* due to ROS induced destruction of chlorophyll stability (Sameena and Puthur, 2021). The chlorophyll content reduction and accumulation of Na and Cu in plant parts in response to NaCl and CuSO_4 stresses cause reduction of CSI in

V. inermis. The significant positive correlation between CSI and chlorophyll content ($r = 0.88$ and $p \leq 0.05$ in NaCl treated plantlets and $r = 0.89$ and $p \leq 0.05$ in CuSO₄ treated plantlets) and significant negative correlation of CSI with bioaccumulation of Na/Cu ($r = -0.99$ and $p \leq 0.01$ in NaCl treated plantlets and $r = -0.99$ and $p \leq 0.01$ in CuSO₄ treated plantlets) compliments the preceding remarks.

5.3.2 Photosystem (PSI and PSII) activities

The photosystem activities (PSI and PSII) are sensitive to environmental stresses. The activity of PSI was found to be reduced under both NaCl and CuSO₄ stresses in *V. inermis* plantlets. Salinity stress has been found to reduce the PSI as well as PSII activities of plants (Gururani et al., 2015). The higher accumulation of Na/Cu in the plant parts cause damages to the thylakoid membrane and thereby reduce the photosystem activities (Shackira and Puthur, 2019; Siddiqui et al., 2022). According to Shackira and Puthur (2019), the degradation of thylakoid membrane protein complex destabilize the thylakoid membrane and it results in the reduction of PSI activity. The significant positive correlation between MSI and PSI activity ($r = 0.97$ and $p \leq 0.01$ in NaCl treated plantlets and $r = 0.99$ and $p \leq 0.01$ in CuSO₄ treated plantlets) as well as MSI and PSII activity ($r = 0.95$ and $p \leq 0.01$ in NaCl treated plantlets and $r = 0.94$ and $p \leq 0.01$ in CuSO₄ treated plantlets) corroborate the finding. Similar to the present study, it was observed by Sameena and Puthur (2021) that higher Cu concentration in the growth medium reduced the PSI activity of castor seedlings.

PSII activity of *V. inermis* plantlets were unaffected under low concentration of NaCl. However, with increasing concentrations of NaCl, the activity of PSII declined. Gururani et al. (2015) has reported that salinity stress is capable enough to reduce the activity of both PSI and PSII. In this present study also the CuSO₄ treatment reduced the activity of PSII.

Similarly, the PSII activity of castor seedlings was decreased significantly under Cu stress (Sameena and Puthur, 2021). The damaged antenna molecules and reaction centers as a result of Cu stress inactivates the PSII donor side which inhibits PSII electron transfer and thereby reducing PS II activity (Gao et al., 2018; Shackira and Puthur, 2019). The significant negative correlation between bioaccumulation of Cu with PSI ($r = -0.98$ and $p \leq 0.0$) and with PSII ($r = 0.98$ and $p \leq 0.01$) clearly indicates the reduction in PSI and PSII activities, due to Cu accumulation. The stability of PSII is also dependent on various enzymes and polypeptides that take part in photosynthetic processes. The degradation of these enzymes and polypeptides by ROS produced as a result of Cu stress affects the stability of PSII and the oxygen- evolving complex negatively (Myśliwa-Kurdziel et al., 2004).

5.3.3 Chlorophyll *a* fluorescence and gas exchange

In studies of plant stress, photosynthetic measurements are an essential component since photosynthesis is highly sensitive to environmental conditions. Traditional approaches, even those that are technically sophisticated, including measuring photosynthetic rates by gas exchange (CO_2 , H_2O , and O_2), take a long time and give only partial information on the overall performance of the photosynthetic system. Contrarily, measurements of chlorophyll *a* fluorescence offer a straightforward, non-destructive, affordable, and quick method for analysing light-dependent photosynthetic processes in plants (Kalaji et al., 2012; 2016). The shape of the induction curves for chlorophyll fluorescence revealed the alterations in the light-dependent photosynthetic process of *V. inermis* under NaCl and CuSO_4 treatments. Chlorophyll *a* fluorescence transient OJIP curves measured in *V. inermis* plantlets treated with lower concentrations of NaCl or CuSO_4 were not significantly impacted. However,

compared to the control plantlets, those subjected to higher NaCl and CuSO₄ concentrations exhibited OJIP curve with dramatic changes. The reduction of initial fluorescence (F_0) in 400 mM NaCl treated plantlets of *V. inermis* may be due to the inefficacy of the reaction centers for energy absorption, which was clearly evident from the reduction in photosystem activities. The reduction in F_0 in *V. inermis* grown under higher NaCl and CuSO₄ indicates the vulnerability of the plant towards Na and Cu stresses (Vassilev and Manolov, 1999). In the current study, plantlets grown in higher NaCl and CuSO₄ concentrations showed decreased F_m when compared to control plantlets. The decrease in F_m under stressful circumstances denotes the dissociation of the PSII light harvesting complexes (Ouzounidou et al., 2003). The reduction in F_m is also due to the decrease of the pigment content in plantlets grown under higher NaCl and CuSO₄ concentrations (Paunov et al., 2018).

The area above the fluorescence induction curve (area) was decreased in plantlets exposed to 300 and 400 mM NaCl and 200 μ M CuSO₄ as compared with control plantlets. The reduced area above the fluorescence induction curve typically indicate the effect of higher NaCl and CuSO₄ on photosynthetic machinery and is linearly proportional to the pool size of electron acceptor such as plastoquinones on the reducing side of PSII. So, the plantlets treated with 300 and 400 mM NaCl and 200 μ M CuSO₄ had reduced photosynthetic rate due to the blockage of the electron transfer from reaction centre to electron acceptor pool (Strasser et al., 2004; Mathur et al., 2016; Sruthi and Puthur, 2020; Janeeshma et al., 2021a). The reduced electron accepters severely affect the photosynthetic electron transport process.

The maximum quantum yield of PSII (F_v/F_m) represents the capturing and conversion efficacy of the light energy and it was reduced in 400 mM NaCl and 200 μ M CuSO₄ treated plantlets. The reduction in

photosynthetic pigment and associated membrane damages affect the quantum yield of *V. inermis*. The stress induced destabilization of PSII and inactivation of D1 proteins severely affect the light capture and quantum yield. The thylakoid membrane damages associated with 400 mM NaCl and 200 μ M CuSO₄ treatments in *V. inermis* also have a role in reduced Fv/Fm. This was clearly supported by the negative correlation between Fv/Fm and MDA ($r = -0.918$ and $p \leq 0.01$) and EL ($r = -0.0.878$ and $p \leq 0.01$). The parameter Fv/Fo represents the maximum efficiency of water splitting complex. The stress induced alteration on water splitting complex was higher in plantlets treated with 400 mM NaCl and 200 μ M CuSO₄. The increased thylakoid membrane damage and stress induced alterations on D1 and D2 proteins cause negative impact on water splitting complex (Sachdev et al., 2021). Similar results were also observed in maize and rice plants under the influence of heavy metals like Cd and Zn (Janeeshma et al., 2021a,b).

The indices such as PI_{ABS}, PI_{total} and SFI indicates plant performance under stress. The higher reduction in these parameters clearly showed the inability of the plantlets to perform better in higher NaCl and CuSO₄ concentrations. The performance indices such as PI_{ABS} and PI_{total} is a measure of the performance index of electron flux to the final PSI electron acceptors (Mehta et al., 2010). The reduction of PI_{ABS} and PI_{total} in response to higher NaCl and CuSO₄ concentrations and increase of the same parameters in lower NaCl and CuSO₄ concentrations specifically validates the deleterious and beneficial effect of lower and higher Na and Cu concentration in the growth of *V. inermis*. Similar observation was found in the plantlets of *Ricinus communis* under Cu stress (Sameena and Puthur, 2021). The N represents the turnover number of Q_A, more specifically the number of times Q_A was reduced from 0 to Tf(max). The N was reduced progressively in plantlets of *V. inermis* in response to NaCl treatments and the reduction was

maximum in plantlets grown in 400 mM NaCl. The multiple turnovers of Q_A reductions (S_m) is related to the efficient PSII centers which have the capacity to transfer an electron from Q_A to Q_B (Antal et al., 2009). Stirbet (2011) concluded that, S_m is the indication of number of electrons passing through the electron transport chain from the donor side of PSII to the acceptor side of PSI. It was drastically reduced in the plantlets subjected to 300 and 400 mM NaCl and the reduction was 35 and 40% respectively as compared with the control plantlets. Similarly, exposure to 200 μ M $CuSO_4$ also resulted in the reduction of S_m and it was 34% lower than control plantlets. However, the influential role of lower concentration of $CuSO_4$ (100 μ M) on plant growth and photochemistry was evident from the increment in S_m and it was 20% higher than control plantlets.

The PSII relative driving force index on absorption basis is represented as DF_{ABS} . The DF_{ABS} was reduced in response to NaCl treatments of higher concentrations (300 and 400 mM). Similarly, 200 μ M $CuSO_4$ modulates the DF_{ABS} negatively and the reduction was 147% as compared with the control plantlets. The higher reduction in DF_{ABS} was found in plantlets treated with higher $CuSO_4$ (200 μ M) and it was higher than any of the NaCl treatments. The values of DF_{ABS} and DF_{total} indicates the role of discrete components in the driving process of whole electron transport chain. The particular reduction in these parameters is due to the phytotoxicity of higher NaCl and $CuSO_4$ concentrations. The photosynthetic yield parameters such as $PHI(Eo)$ [ϕEo], $PSI(Eo)$ [ΨEo], and $PHI(Po)$ [ϕPo] represents quantum yield (at $t = 0$) for electron transport from Q_A^- to plastoquinone, the probability (at $t=0$) that a trapped exciton moves an electron in the electron transport chain beyond Q_A^- and the maximum quantum yield of primary PSII photochemistry at $t = 0$ respectively. All photosynthetic yield parameters showed reduction in higher concentrations of NaCl and $CuSO_4$ where as it was less affected in plantlets treated with

lower concentrations. The reduction in the quantum yield ($\Phi(\text{Eo})$) was allied with the inhibition of electron transfer to PQ (Li et al., 2010).

The phenomenological energy pipeline models indicate the flow of energy and the related photochemistry of *V. inermis*. The phytotoxicity of the NaCl and CuSO_4 increased with the increasing concentration and accordingly cause the reduction in the ABo/CSm, TRo/CSm and ETo/CSm. The decrease in the absorption and trapping of light energy and thereby, electron transport was due to the increase in the inactive reaction centres. The reduction in the functional reaction centres leads to the reduced photosynthetic efficiency. The decrease in the amount of energy absorption by chlorophyll molecules is due to a drop in PSII antenna size or a structural change in minor antenna LHC component (Tongra et al., 2011). The distribution frequency of active reaction centres in a cross-sectional area was lower in response to higher NaCl and CuSO_4 and it was reflected through the reduction in ABo/CSm. The dissipated energy flux per CS approximated by Fm is represented as DIo/CS_M. The plantlets grown in 400 mM NaCl and 200 μM CuSO_4 exhibited an increase in DIo/CS_M as compared to the control plantlets. The enhancement in DIo/CSm of *V. inermis* plantlets was due to the ineffectiveness of the reaction centres to effectively utilize the absorbed light energy for electron transport. The increase of inactive reaction centres and the reduced electron transport due to the reduction of functional Q_A pool causes an increment in DIo/CSm (Force et al., 2003; Kalaji et al., 2011; Faseela et al., 2020).

5.4 Osmolality and osmolytes

The maintenance of the osmotic potential for the purpose of normalized water status in plants subjected to abiotic/biotic stresses have much importance. The presence of salinity and toxic metals in the growing habitats of plantlets severely affects the water uptake by the roots

(Kholodova et al., 2011; Rucińska-Sobkowiak et al., 2013). The reduced uptake of the water can have negative influence on plantlet's growth and metabolism. But at the same time, the alterations in the water transport dynamism in response to salinity and heavy metals functions as a regulatory mechanism for the avoidance of the toxic metal influx into the plant biomass.

To counter the shortage of water in the cells, plants synthesise several osmolytes within the cells. The enhanced synthesis of compatible solutes is associated with the plant tolerance towards the metal (Anjum et al., 2016b). As a survival strategy, plants frequently reduce their biomass in response to stress, which is linked to the inability to assimilate carbon (Sarath et al., 2024). Moreover, plants allocate more of their assimilated carbon towards the energy required to maintain normal cell activities including the maintenance of water potential (Pompeiano et al., 2017; Asrar et al., 2018).

Halophytes can endure high salt and heavy metal concentrations by adopting mechanisms that operate at the cellular level, which significantly contributes to the defense system. These mechanisms include osmotic adjustment, ion homeostasis, enzymatic or nonenzymatic antioxidant defense systems, and modifications of the membrane components (Mansour and Salama, 2019). The maintenance of osmoticum under stressful environment and shielding the subcellular organelles has been considered as the key regulatory mechanisms in plant stress physiology (Suprasanna et al., 2016). As part of NaCl tolerance, the toxic Na⁺ is sequestered into the vacuole, resulting in the solute potential difference between the vacuole and the cytoplasm. This conflict in the solute potential can be neutralized by the accumulation of osmolytes in the cytoplasm. The NaCl-treated plantlets showed a higher accumulation of sugars, proline, amino acids and proteins, which can function as compatible solutes. The increase in osmolality of roots

and leaves due to NaCl treatment compared to control plantlets supports the aforementioned claims (Sarath et al., 2024). The enhanced accumulation of osmoprotectants safeguards the organelles, bio-membrane, and cytosolic enzymes under stress conditions (Debnath et al., 2018). However, it has been demonstrated that the accumulation of compatible solutes during stress conditions inhibits plant growth (Choudhary et al., 2023). Palliyath and Puthur (2018) reported that significant accumulation of sugars occurs in the halophytic plant *Bruguiera cylindrica* in response to NaCl stress. The augmented level of sugars in NaCl treated plants mainly contributes towards the build-up of the osmoticum of the cell sap.

Sruthi and Puthur (2019) observed an increase in the osmolality of leaves and roots of *Bruguiera cylindrica* under Cu stress. In the case of *Oryza sativa* plants under Cd and Zn stress also, an increase in osmolality was observed (Janeeshma and Puthur, 2023). A similar increase in osmolality was found in the leaves of *Ricinus communis* seedlings under CuSO₄ exposure (Sameena and Puthur, 2021). This corroborates with our results wherein, it was observed that there was concentration dependent increase in the osmolality of leaves and roots of *V. inermis*. Increased osmolalities is attributed to the accumulation of free amino acids, sugars and other compatible solutes in the plants (Sameena and Puthur, 2021). Elevated osmolality reduces the wilting point, thereby reducing the stress effects in plants (Bartlett et al., 2012a,b; Charrier et al., 2013).

Higher proline content is essential for osmoregulation, ROS abatement, and the stabilization of cellular components. In addition to acting as osmoprotectants, the increase in proline and total amino acids concentration also serves as a source of nitrogen and carbon. Consequently, it regulates nitrogen availability under high-stress conditions (Iqbal et al., 2014; Vaishnav et al., 2016). Proline also play key roles in redox buffering,

acting as molecular chaperones and maintaining the integrity of membranes and proteins (Verbruggen and Hermans, 2008; Jisha and Puthur, 2014). On exposure to NaCl, the proline content in the leaves and roots of the treated plantlets gradually increased. In comparison to the roots, the increase was greater in the leaves. Being the site of synthesis there is more probability of greater accumulation in the leaves. Proline has a significant capacity for hydration and therefore its hydrophilic part can bind to water molecules, whereas its hydrophobic terminus can bind to proteins. Proteins containing proline can bind more water, avoiding dehydration and denaturation in response to osmotic stress (Liang et al., 2013). Verslues and Sharma (2010) reported that, enhancement in proline accumulation occurs in plants under salinity stress and later it is broken-down during the recovery of the plants from stress and this act as a carbon and nitrogen reservoir, aiding the plants to recover from stressed condition.

The higher proline content in the leaves significantly correlate with glutathione content in *V. inermis*. The significant positive correlation between glutathione with proline content in leaves ($r = 0.972$ and $p \leq 0.01$ in NaCl treated plantlets and $r = 0.958$ and $p \leq 0.01$ in CuSO₄ treated plantlets) and roots ($r = 0.985$ and $p \leq 0.01$ in NaCl treated plantlets and $r = 0.993$ and $p \leq 0.01$ in CuSO₄ treated plantlets) strongly supports the same. The higher synthesis of proline itself act as a feedback inhibitor of the enzyme glutamate kinase, which is a part of the bifunctional enzyme P5CS (Pyrroline-5-carboxylate synthase). The inhibited glutamate kinase results in the increased glutamate content in the plant cell paving way for the enhanced synthesis of glutathione. This glutathione act as a structural component of phytochelation in plant cell which help to chelate toxic heavy metals (Pavlikova et al., 2008). Moreover, higher amount of proline in chloroplast under an abiotic stress environment helps to maintain a lower NADPH: NADP⁺ ratio, which can be helpful in electron flow between photosynthetic

excitation centres, redox homeostasis and also play a vital role in the reduction of photoinhibition and protect the photosynthetic apparatus (Hare and Cress, 1997; Acosta et al., 2017). Khedr et al. (2003) reported that, high amount of proline under NaCl stress in the halophyte *Pancreatium maritimum* play an important role in salinity tolerance by maintaining the enzymatic property of important enzymes and by stabilising the detoxifying enzymes and indirectly help in the synthesis of stress induced protein. The higher synthesis of proline on exposure to 0.25mM CuSO₄ in *Bruguiera cylindrica* was reported by Sruthi and Puthur (2019). The significant synthesis of proline in different rice cultivars on exposure to 6 mM ZnSO₄ and 2 mM CdCl₂ were reported by Sinisha and Puthur (2018). The increase in proline content during Cd and Zn stresses in rice cultivars help them to survive in the extremity and also proline has a role in forming proline-metal complex helping the plants to chelate the metal in the roots.

There was considerable increase in total soluble sugars content of *V. inermis* in response to increasing concentration of NaCl, and it was higher in the leaves than roots. During stressful conditions, increased sugars serve as a carbon reserve source in addition to their function as an osmoprotectant. Therefore, the increased total soluble sugar content indicates a plant's tolerance potential to higher salinity (Dogru and Ecem Bayram, 2016). The augmented level of sugars in leaves of NaCl-stressed *V. inermis* plantlets can also be related to the restriction of sugar transport to storage organs and developing tissues (Krasensky and Jonak, 2012). Chai et al. (2014) and Sruthi and Puthur (2019) found enhanced total soluble sugar content in *Spartina alterniflora* and *Bruguiera cylindrica* respectively under Cu stress. Han et al. (2012) observed increased total soluble sugar content in *Kosteletzkya virginica* during the first two weeks of Cu treatment after which it decreased. Cu stress was found to improve the total soluble sugar content in leaves of *Helianthus annuus* and *Cucumis sativus* (Alaoui-Sosse et al., 2004; El-Tayeb et

al., 2006). Maize cultivars (cv. Pegaso and cv. Akpınar) and barley seedlings also had enhanced sugar content under Cu stress (Guzel and Terzi, 2013; Kalai et al., 2014). Other heavy metals like Cd also was found to enhance the total soluble sugar content in halophytic plants like *Atriplex halimus* (Lefèvre et al., 2010b). The accumulation of total soluble sugars in barley plants increased under single heavy metal stress (Cu) and multiple heavy metal stress (Cu, Al and Cd) (Guo et al., 2007). The increased accumulation of sugars act as a defensive response against the production of ROS in order to stabilize the membranes of plant cells (Keunen et al., 2013). But a study on the halophyte *Acanthus ilicifolius* conducted by Shackira and Puthur (2016) depicted decreased total soluble sugar content in response to Cd which was due to the detrimental effect of Cd on carbohydrate metabolism of plants.

Proteins are the macromolecules which are functionally diverse and have definite roles in plant growth and metabolism. They have definite role in cellular signalling, catalysis (as enzymes), transport of molecules, membrane fusion, structural support, and stress amelioration. The modulations including up/down regulation in the synthesis of proteins depends on the conditions in which they survive. The disruption of the bonding and misfolding of already existing proteins and stress induced expression of proteins were reported in plant cells under stress conditions including NaCl and heavy metals (Hasan et al., 2017; Sruthi and Puthur, 2019; Sarath and Puthur, 2021). The protein peroxidation leads to the increase of amino acids content of plant cells. In the present study, the soluble protein content of the leaves and roots increased in response to the NaCl treatment as compared to control plantlets. The increase was more prominent from 10d onwards in both leaves and roots. The higher accumulation of soluble protein was found in the leaves as compared to the roots. There was a minor decrease in soluble protein content in the plantlets treated with 100 mM NaCl as compared to the control plantlets.

5.5 Free radical scavenging mechanisms

5.5.1 Enzymatic antioxidants

Plant has a very strong defensive mechanism to encounter the oxidative stress due to biotic or abiotic factors. The scavenging of ROS and maintaining the cellular redox homeostasis is the prime function of the free radical scavenging machinery. For strong counteraction against oxidative stress, plants have two major mechanisms, which include enzymatic and non-enzymatic antioxidants. The non-enzymatic antioxidants include, ascorbate, glutathione, phenolics, flavonoids and alkaloids etc. which independently or in coordination with enzymatic antioxidants such as superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, and ascorbate peroxidase reduce or inhibit the cellular damage caused due to ROS (Laxa et al., 2019). The first line of defence in plants was done by the enzyme superoxide dismutase. It has a vital role in the conversion of toxic $O_2^{\cdot-}$ to H_2O_2 . The downstream detoxification of H_2O_2 to non-toxic water and oxygen was taken care by catalase, peroxidase etc (Biczak, 2016; del Rio et al., 2018). Salinity has been shown to boost the activity of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, and ascorbate peroxidase, which improves ROS scavenging (Garratt et al., 2002; Gill and Tuteja, 2010; Afridi et al., 2019; Altaf et al. 2023).

The present investigation also showed increased activity of the enzymes SOD, POD, and APX when plantlets were exposed to NaCl. The increment in the activities of antioxidant enzymes in the plantlets exposed to higher concentrations of NaCl and lesser activities in plantlets treated with lower concentrations of NaCl clearly indicates the stress induced induction signal for the reduction of oxidative stress and it was easily correlated with the ROS generation in *V. inermis*. Sruthi and Puthur (2020) found that the

tolerance capacity of *B. cylindrica* under extreme concentrations of NaCl (400, 500 and 600 mM) was mainly due to the higher activities of the antioxidant enzymes such as POD, APX and SOD and they suggested that increased level of these enzymes activities can be a useful biomarker to determine the salt tolerance potential. Similar observation was also reported by Shackira and Puthur (2016) in their study on halophyte *A. ilicifolius* exposed to NaCl stress. The antioxidant enzymes such as SOD, POD and APX exhibited enhanced activities in plantlets exposed to 400 mM NaCl but at the same time there was a reduction in activities of these three enzymes in plantlets treated with 600 mM NaCl. They concluded that, the concentration above 400 mM NaCl, was intolerable to *A. ilicifolius*. The exposure of NaCl caused a significant increase in SOD activity in two mangroves, *B. gymnorrhiza* and *B. parviflora* (Takemura et al., 2000; Parida et al., 2004b). Cai-Hong et al. (2005) reported that higher salinity induces the effective ROS scavenging mechanism in the C3 halophyte *Suaeda salsa* and it results in the reduction of ROS by up regulating antioxidant machinery including ASA, GSH, GR and APX.

The activities of antioxidant enzymes were observed to be higher in *V. inermis* plantlets subjected to 200 μ M CuSO₄ as compared with the control plantlets. Similarly, the activities of the antioxidant enzymes SOD, APX and CAT were found to increase under Cu stress in *Trigonella foenum-graecum* (Elleuch et al., 2013). Also, Cu stress resulted in enhanced activity of the enzymes SOD, CAT and POD in *Arabidopsis thaliana* (Drażkiewicz et al., 2004). The increased activities of antioxidant enzymes have been reported in many halophytes exposed to heavy metal stress. Higher antioxidant enzyme activities were also reported in halophytes like *Kosteletzkya virginica*, *Salicornia brachiata* and *Spartina alterniflora* by Han et al. (2013), Sharma et al. (2010a) and Chai et al. (2014) respectively under heavy metal stress. A sharp increase in the activities of SOD, POD and CAT were reported in *K. candel* in

response to 20 mg L⁻¹ CuCl₂ and it help the plant to sustain in the higher Cu concentration (Rahman et al., 2012). SOD activity increased in the halophyte *Acanthus ilicifolius* under Cd stress (Shackira and Puthur, 2017). An increase in the activities of the enzymes GPX and SOD were reported in *Sonneratia apetala* under Cd stress (Zhang et al., 2010b). The increment in the enzymatic antioxidants in *V. inermis* under NaCl and CuSO₄ stress help them to cope up under these severe situations and thereby showed higher tolerance against NaCl and CuSO₄.

5.5.2 Non-enzymatic antioxidants

The most significant non-enzymatic ROS scavenging antioxidants, such as ascorbic acid, glutathione, flavonoids and phenolics, play a crucial function in the defense system of plants by preventing the oxidative stress. Increased synthesis of total phenolics, ascorbate, glutathione and flavonoids were found in *V. inermis* in response to NaCl and CuSO₄ stress. In leaves of plantlets treated with 300 and 400 mM NaCl, the total phenolics content exhibited a pronounced increase (5 and 7-fold) as compared to control plantlets. Phenolic compounds have most ideal structural chemistry to detoxify ROS. The phenolic compounds is a class of secondary metabolites, including flavonoids, tannins, lignin and hydroxycinnamate esters. The high reactivity of polyphenols as hydrogen or electron donors and the capacity of the radical produced from polyphenols to stabilize and delocalize the unpaired electron (a chain-breaking mechanism) are highly useful in ROS scavenging, and it effectively functions in plants during oxidative stress (Rice-Evans et al., 1997; Ahmad et al., 2010).

The phenolic compounds have the potential to interfere with the oxidation of membrane lipids and other compounds by rapidly contributing their hydrogen atom to radicals, producing phenoxy radicals. These are stable intermediates and cannot initiate any other reactions. Thus they also

serve as terminators of many prolonged chain reactions by intermingling with free radicals. In the case of Cu stress (redox active metal), the highly augmented phenolic compounds act as pro-oxidants and participate in H₂O₂ scavenging (Ahmad et al., 2010; Sachdev et al., 2021). A significant increase in phenolic compound was found in the halophyte *Cakile maritima* on exposure to NaCl and they conclude that, the increase in polyphenol content contribute towards increased antioxidative potential under NaCl stress (Ksouri et al. 2007). Studies conducted in three halophytic plants (*Arthrocnemum macrostachyum*, *Sarcocornia fruticosa* and *Salicornia europaea*) exposed to different concentrations of NaCl ranging from 0 to 600 mM exhibited an increase in phenolic content and it increased upto 400 mM NaCl and there after a decrease occurred (Ghanem et al., 2021). These observations also validate the present study, wherein both leaves and roots accumulated significant amount of phenolics content on exposure to CuSO₄. The maximum accumulation was found in the leaves compared with that of roots. The increase in total phenolics content was 167 and 187% (in leaves) and 71 and 48% (in roots) in plantlets treated with 100 and 200 µM CuSO₄ on 20d as compared with that of control plantlets. The increase of phenolics content attributes metal chelating efficiency to plantlets and help in the formation of metal-phenolics complex in the plant biomass (Chauhan et al., 2017).

Glutathione, a well-known tripeptide (γ-glutamylcysteinyl-glycine) was specifically over expressed in plants under abiotic or biotic stresses. The main source of nonprotein thiols in the majority of plant cells is glutathione. The plantlets exposed to NaCl recorded an increase in the content of glutathione in leaves and roots and it increased with increasing concentration of NaCl stress. A similar trend of increase was also found in the root glutathione content and the higher accumulation was on 20d of NaCl treatment. The CuSO₄ treatment also cause an increment in glutathione

content on 20d and it was higher in roots than leaves. The stress induced induction of glutathione is very well suited to perform a wide variety of biological functions in all organisms due to the chemical reactivity of its thiol group. The thiol group's nucleophilic character is crucial for the formation of mercaptide bonds with metals and for its reactions with particular electrophiles (Ahmad et al., 2010). Multiple ways exist for glutathione to act as an antioxidant. In the ascorbate-glutathione cycle, GSH is involved in reducing DHA (dehydroascorbate). Glutathione reductase uses NADPH as the reducing power to catalyse the regeneration from GSSG to GSH. The high reductive potential of GSH is owing to a core nucleophilic cysteine site. It neutralizes harmful H_2O_2 and engages in non-enzymatic reactions with singlet oxygen, superoxide radicals, and hydroxyl radicals (Millar et al., 2003). The higher synthesis of glutathione may also help in the chelation of metal ions within its roots. The higher synthesis of PCs, the thiol-rich family of proteins having general structure (γ -Glu-Cys) n-Gly, are derived from glutathione (GSH) and have the capacity to chelate and detoxify the metals (Cobbett and Goldsbrough, 2002; Seregin and Kozhevnikova, 2023).

Ascorbate plays a crucial part in detoxification of H_2O_2 and other harmful oxygen derivatives in the plants. Ascorbate fundamentally functions as a reductant and scavenges a wide variety of free radicals. In the ascorbate-glutathione cycle, APX uses two molecules of AsA to reduce H_2O_2 to water, producing monodehydroascorbate. The monodehydroascorbate can be reduced straight into ascorbate. In severe stress condition, the higher proportion of ROS in cells destroy the redox homeostasis. To care of this, the non-enzymatic oxidant like ascorbate has crucial role to play and this was observed in the present study. Higher level of ascorbate was found to accumulate in *V. inermis* in response to NaCl and CuSO_4 treatments. In *V. inermis* there was a progressive increase in ascorbate content upto 300 mM NaCl concentration and further it gets reduced and maintains as like that of

control plantlets. The reduction of ascorbate in higher concentration (400 mM NaCl) could be due to irreparable damages in the biosynthesis pathway of ascorbate. The studies conducted in *B. cylindrica* clearly proved that the ascorbate level was dramatically dropped under 600 mM NaCl stress, but at the same time there was no significant decrease at 500 mM, showing that the production of ascorbate was not adversely impacted by 500 mM NaCl concentration (Sruthi and Puthur, 2020). The lower ascorbate content in *V. inermis* exposed to higher concentrations of NaCl was consistent with the findings stated above.

Another class of compounds called flavonoids are generally synthesised in the plant body to effectively encounter oxidative stress. It has the ability to reduce the ROS by effective scavenging mechanism and also help the process of chelation mainly in the case of transition metals (Cu, Fe etc). In our investigation, the NaCl and CuSO₄ treatments led to an increase in flavonoid content showing the potent ROS scavenging potential of this secondary metabolites. The flavonoids actively cooperated with other antioxidants to maintain a proper redox equilibrium (Losada-Barreiro et al., 2022). In salt-sensitive rice varieties compared to salt-tolerant types, the gene flavanone 3-hydroxylase (F3'H), which is involved in the production of antioxidant flavonoids, was expressed increasingly (Walia et al., 2005). The presence of excess copper in root suspension culture of *Lemna gibba* has also increased flavonoid content (Luteolin-glycosides) and free radical detoxification potential (Babu et al., 2003; Ali et al., 2006). The accumulation of flavonoids with the highest antioxidant capacity was positively correlated with an upsurge in ROS concentration brought on by stress. Likewise, in this study also the CuSO₄ exposure positively influenced the flavonoid biosynthesis and the increase was maximum in higher CuSO₄ treatment (200 µM).

5.6 Bioaccumulation and translocation of Na and Cu

The plants which are adapted to grow in high salt concentrations modulates its physiology to cope up with severe osmotic stress. Mangroves and mangrove associates are plants that are capable of growing under high saline conditions. These plants employ various strategies like salt exclusion (salt avoidance) by selectively restricting the uptake of salts or by salt accumulation wherein the uptaken salt is sequestered inside the vacuole so as to avoid toxicity. The present study witnessed a high accumulation of Na in the halophyte *V. inermis*. With increasing concentrations of Na in the medium, the plant accumulated increasing concentrations of Na in its tissues. This occurs probably by intracellular compartmentation wherein majority of the ions are sequestered in vacuoles. According to Munns et al. (2006), the absorbed excess Na ion was, sequestered in the pericycle, xylem parenchyma cells, root cortex (particularly the casparian strip), and phloem cells. On the other hand, salt-accumulating halophytes effectively compartmentalize ions inside the vacuoles and accumulate appropriate solutes in the cytoplasm to counteract the toxicity, conferring salt tolerance (Gorham, 1995).

The higher accumulation of Na⁺ in *V. inermis*, is due to the proper sequestration of Na⁺ ions in the vacuole and other parts of the plants. This was clearly evident from the subcellular distribution studies of Na⁺. A remarkable higher accumulation rate was detected in the soluble fraction of tissues containing ribosomes, vacuoles and cytoplasm over the control. Vacuolar Na⁺ sequestration is an important and cost-effective osmotic adjustment method that also lowers Na⁺ concentration in the cytosol (Parida and Jha, 2010b). The sequestration of Na⁺ and Cl⁻ into the vacuoles of the hypodermal storage tissue of leaves results in salt accumulation in halophytes (Kura-Hotta et al., 2001). It has been demonstrated that if the

harvested biomass of hyper salt accumulators' shoot part is not returned to the same soil, it can considerably contribute to the removal of salts from salt lands and also serve as a useful amelioration processes in desalination process (Owens, 2001). Similar studies conducted on *Haloxylon recurvum*, *Atriplex halimus*, *A. amnicola*, *A. calotheca*, *A. hortensis*, *Chenopodium album*, *Salsola kali*, and *Suaeda nudiflora* also reported increased Na concentration in the plant as a result of increased salinity in the medium (Khan and Aziz, 2001; Shekhawat et al., 2006; Belkheiri and Mulas, 2013). Other halophytes like *Lobularia maritima*, *Plantago maritima* and *Tripolium pannonicum* have also been reported to accumulate concentrations as high as 50-500 mM NaCl probably due to the above-mentioned reason (Popova and Golldack, 2007; Sleimi et al., 2015; Ludwiczak et al., 2023).

Plants that are tolerant to salinity may also be capable of coping up heavy metal toxicity (Amaria and Brahim, 2016). *V. inermis* plants subjected to Cu stress were found to accumulate high levels of Cu in its tissues. As the concentration of Cu in the medium was increased, the plant was found to accumulate increasing levels of Cu in various tissues with the maximum accumulation in the root tissues. Similar observations were made by Sameena and Puthur (2021) in *Ricinus communis*. The present study also witnessed decreased translocation of Cu to the shoots of *V. inermis*. The plant specific mechanism of tolerance against heavy metals varied in accordance with the plant species. The stabilization of Cu in the root tissues due to lower translocation to shoot help the plants to tolerate severe Cu stress in leaves and thereby, protect the photosynthetic machinery. According to Marques et al. (2018), Cu strappingly binds to the root cell wall and thereby restrict its movement to shoot system. The restriction of Cu in the root tissue was statistically calculated by TF. The TF of the CuSO₄ treated plantlets were very low and it was even below 1 (0.096 ± 0.004) in plantlets exposed to higher CuSO₄ treatment. The sequestration of Cu in the cell wall

of *V. inermis* was evident from the subcellular distribution studies. In the case of CuSO_4 treated plantlets, the cell wall and cell wall debris fraction of the leaf, root and stem showed higher accumulation of Cu. Among them, the highest accumulation was found in the cell wall and wall debris of the root tissue.

5.7 Subcellular distribution

The subcellular distribution pattern of elements such as Na, K, and Ca indicates that the vacuole acts as a sink for these ions. This suggests that the detoxification of excess Na^+ ions in the NaCl treated *V. inermis* was facilitated to a greater extent through the vacuolar sequestration without causing much deleterious effect on other active parts of the cell (Sarath et al., 2024). In addition, it was also noted that the concentration of Ca increased in fraction 4 (ribosomes, vacuoles, and cytoplasm), demonstrating the inseparable role of Ca in helping in the vacuolar sequestration of excess Na^+ ions and thereby imparting tolerance to NaCl (Seifikalhor et al., 2019). Ca signaling in response to salt stress has been studied by various groups, and have found that salt overly sensitive (SOS) signaling triggered by Ca is a common amelioration mechanism seen in stress-tolerant plants. In the current study, a prominent peak was observed in the subcellular distribution level of Ca, especially in the soluble fraction of leaf tissue, where the maximum allocation of Na was observed (Sarath et al., 2024). Hence, the rapid increase in the concentration of Ca in response to the NaCl exposure indicates the upregulation of signalling mechanisms necessary for the coordination of the defense strategies to maintain the ionic balance and phenotypic plasticity under stressed conditions, as suggested by Manishankar et al. (2018).

Interestingly, the uptake of Na was correlated with the increased level of K in the fraction containing ribosomes, vacuoles, and cytoplasm, which

might be due to the structural similarity of both ions, as suggested by Fakhrfeshani et al. (2015). Moreover, K effectively counters NaCl-induced stress by mediating various enzymes' activities (Benito et al., 2014). Hence the maintenance of the Na/K ratio, along with other ions like Ca, acts as a reliable indicator for NaCl stress tolerance in plants, as suggested by Guo et al. (2022). From the results of ionic allocations, it can be concluded that maintaining ionic homeostasis is a versatile strategy operating in salt-tolerant plants to cope with adverse conditions (Sarath et al., 2024).

The Cu ions uptaken was increasingly complexed with the cell wall as evidenced by the results of subcellular localisation of metal ion. This was in accordance with the earlier studies in *Ricinus communis* where the Cu ions were found to be complexed with the polysaccharides of cell walls and thereby, limiting the cellular toxicity to a greater extend (Ren et al., 2020). As roots are the first organs to come in contact with the toxic metal ions, root cell wall prevents the entry of metal ions and create a first level of barrier. Root cell wall consists of several polysaccharides such as pectin, hemicelluloses, cellulose and glycoprotein and are potential in binding toxic metals and complex them in the cell wall as it contains many functional groups, such as -COOH, -OH and -SH (Colzi et al., 2012; Ren et al., 2020). Hence the increased accumulation of Cu ions observed in the root cell wall is a first line of defence system operating in *V. inermis* in response to excess Cu ions.

Plant vacuole constitute a vital organelle in the detoxification process as it sequesters the harmful ions within and thereby maintains the cytosol in safer zone. In the present study the excess Na⁺ ions were found to be stored in the vacuole than other cellular fractions which might be an ideal mechanism to deal with the nutrient imbalance and ion homeostasis under stressed conditions (Mansour, 2023). Likewise, the influx of Ca and K ions

across mitochondrial membrane is relevant for ensuring the bioenergetic performance under stressed conditions (Carraretto et al., 2016). Hence the increased concentration of Ca and K ions in the mitochondrial fractions might be an adaptive strategy operating in *V. inermis* under Cu toxicity so as to meet the energy demand.

5.8 Ionomics of different plant parts

The presence of excess salt near the rhizosphere exerts a severe influence on absorption of mineral ions that have multifaceted functions and plays crucial roles in imparting stress tolerance in plants. Recently, several studies have been carried out depicting the mechanism behind various mineral ions' uptake, accumulation, transportation, and functioning under stress conditions. It is quite natural that exposure to NaCl may enhance the uptake of Na in the tissues of *V. inermis*, and the content of the same was increased in the order root>stem>leaf. This indicates the limited translocation of the uptaken Na⁺ to the shoot system (Sarath et al., 2024). Moreover, the efficient management of ionic balance is mediated by the action of several genes, including Salt Overly Sensitive 1 (SOS1), which can limit the excess accumulation of Na⁺ ions in the shoot system (Guo et al., 2022). Even though the rate of ion accumulation in the shoot system is limited, the transported ions are effectively sequestered in the vacuole, thereby limiting the cytosolic concentration of Na⁺ ions.

Salt stress-induced condition may trigger various ionic imbalance in plants. In the current study, the increased accumulation of Na⁺ negatively affects the uptake rate of other ions, such as Ca and Fe, as suggested by Sagervanshi et al. (2021). This was evident in the case of Ca ions, wherein the rate of Ca accumulation was significantly reduced as the rate of Na increased. Likewise, the uptake of Fe was also negatively affected by the increased accumulation of Na in the roots of *V. inermis*. This might be due to

the enhanced synthesis of organic acids in response to NaCl treatment which complex with the Fe and Ca ions making it less available in free form (Jiaxin et al., 2022). This was further confirmed by the reduced allocation of other nutrients like K, Mg, Mn, Ni, Cu, Zn, Mo, Cr, and Al, along with a prominent peak in the accumulation of organic acids seen in NaCl-treated plants of *V. inermis* (Sarath et al., 2024).

On the other hand, Se was remarkably enhanced in the stem and root tissues of *V. inermis* subjected to NaCl stress. This might be aiding the plant to cope with the oxidative damage induced by NaCl, as suggested by several authors (Feng et al., 2013; Diao et al., 2014). Moreover, it has been proved that supplementing Se to plants under salinity stress greatly enhances the tissue water status, maintains the cell membrane integrity, increases the photosynthetic yield, and enhances the antioxidant activity, which will ultimately enhance the stress tolerance level of the plant to a greater extend (Astaneh et al., 2019).

When plants are exposed to noxious elements like heavy metals, the ionic homeostasis is regulated in order to tolerate the toxic conditions. Based on the difference in the outside environment, plants exhibit various ionic characteristics (Baxter, 2010). Heavy metals have been found to change the concentration of various nutrients in plants. In this study, the concentration of macronutrients (K, Mg, and Ca) tends to have drastic variations in all the tissues i.e., leaf, stem and root subjected to CuSO₄. The increased amount of Ca in *V. inermis* play a key role in signalling under stress. When *Phaseolus vulgaris* plants were exposed to arsenite, N, P, K, Ca, and Mg concentration in the plants increased (Carbonell-Barrachina et al., 1997). But the same heavy metals reduced the absorption of K, Ca, Mg, B, Cu, Mn, and Zn in *Lycopersicum esculentum* (Barrachina et al., 1994). Cong and Ma (2005) observed that at low concentrations of heavy metal, the P and

K concentrations in the fronds of *Pteris vittata* increased. Hua et al. (2022) observed that the uptake of nutrients N, P, K, Mg, Ca, Fe, Mn, Cu, Zn and B in wheat was reduced under low Fe stress in wheat plants. Similarly, a study by Júnior et al. (2014) observed imbalance in the homeostasis of Fe, P, Mg and Mn in sunflower under Cd stress. So, it is a well established fact that based on the heavy metal stress imparted, there is variation in the ionic characteristics of *V. inermis* and it is a strategy adapted to cope up with the particular stress.

5.9 Carbon to nitrogen and carbon to hydrogen ratio

Plant growth and development is regulated by the ratio of carbon (C) to nitrogen (N) metabolites and when the plants are exposed to any stress factors like salinity or drought there may be an imbalance in the C/N ratio (Coruzzi and Zhou, 2001; Chen et al., 2015b). In general, NaCl stress induces ionic imbalance by interfering with various metabolic activities in the cell like N metabolism. In the present study carbon allocation was decreased significantly under abiotic stress conditions, i.e., salinity stress and heavy metal stress (CuSO_4). This limited carbon flux might be due to the diminished rate of photosynthesis, limited stomatal conductance as well as restricted uptake of CO_2 under stressed conditions as suggested by Li et al. (2021).

Likewise, abiotic stress like salinity induces nutritional imbalance by interfering with the nitrogen metabolism leading to reduced growth. It was earlier reported that the uptake of N was limited under salt stress and may negatively affect the N metabolism under stress (Zhang et al, 2023b). The reduced N content observed in the leaf tissue might be due to the decreased translocation of N to the shoot. Since an increased accumulation of N was observed in the root tissue, the uptake of N as the whole was not much affected adversely when the plants were subjected to salt stress. Similar

reduction in N content was observed by Sha (2018) in the leaves of rice plants when exposed to salinity stress.

5.10 Fourier transform infrared spectroscopy

The alterations in the functional groups associated with various carbohydrate structures, phenols, or aldehydes of leaves and roots in response to NaCl and CuSO₄ exposure in *V. inermis* was determined by FTIR. The CH₂ stretching vibration of polyphenols, carbohydrates and proteins in response to NaCl was clear from the peak 2929 cm⁻¹. The absorption peak at 2352 cm⁻¹ represents C=C and it possibly indicates C=C of lignin. It was not found in the plantlets treated with 100 mM NaCl. The higher accumulation of Na lead to the higher synthesis of lignin in the plant biomass. It was also clear from the anatomical studies also. The sequestration of Na and Cu in the cell wall of leaves and roots of *V. inermis* was clearly evident from subcellular distribution studies. The functional groups of cellulose, hemicellulose and pectin act as binding sites of Na/Cu. The strong and broad absorption peak at 3427 cm⁻¹ represents the -OH functional group of alcohols, carbohydrates, proteins and polyphenols. This specific band was widened and shallow in the roots of NaCl treated plantlets (Suresh et al., 2016). Sruthi and Puthur (2019) reported that, -OH and -COOH group has specific role in the sequestration of Cu in the roots of *B. cylindrica*. By the process of chemisorption, the plant forms complex with absorbed metals ions using these functions groups and it help them to stabilize itself in the roots and prevent their translocation to the shoots (Kang et al., 2015). The FTIR data of *Tetraena qataranse* by Panda et al. (2007) revealed that metals like Cr, Cu, Cd and Ni forms a binding interaction with functional groups such as -OH, -COOH, amide, and phosphate groups in roots and help in phytostabilization.

The strong band at 1648 cm^{-1} indicates C-O stretch (amides), N-H bend (nitro compounds, amines), C=O stretch (carboxylic acids, ketones), C=C (alkenes, benzenes) (proline), C=O amide I band and C=O stretching of alkenes. This peak was narrow and sharp in treatments with higher CuSO_4 concentration and more widened and shallower in others. This indicates the interactions of this specific functional group with other molecules. A weak and specific band was observed at 1397 cm^{-1} and denotes CH and CH_2 aliphatic bending group. A characteristic peak was observed in 1255 cm^{-1} representing C-N amide III band and it showed an upregulation of N containing aromatic compounds in plantlets in response to NaCl and CuSO_4 . This characteristic peak was absent or negligible in control and was distinct in CuSO_4 treated plantlets. In a study conducted in *R. communis* seedling by He and Ding (2020) suggested that the sequestration of Cd and Zn in the cell wall is associated with -OH, -NH and -COOH groups. The same functional groups were also detected in *V. inermis* in response to higher NaCl and CuSO_4 . The strong peak at 1055 cm^{-1} represents the S=O stretching (sulfoxides), C-N stretch (amines), C-O stretch (esters, ether, alcohol), =C-H bend (alkenes) (rhamnogalacturonan). The sulfoxide has role in stress tolerance and S=O stretching in CuSO_4 treated plantlets exhibits the same and =C-H bend (alkenes) typically showed the presence of rhamnogalacturonan which indicate the presence of pectin deposition under stress.

5.11 Anatomical modifications

There was no drastic characteristic changes or damages in the anatomy of the leaves of *V. inermis* subjected to 400 mM NaCl. This clearly indicates its tolerance and maintenance of cellular integrity under NaCl stress. The presence of waxy cuticles covered with epidermal hairs prevents water loss. Furthermore, we observed that plantlets exposed to NaCl had

more glandular trichomes. Several studies have shown that trichome density increases in response to environmental challenges, including salinity and drought (Sarath et al., 2024). It was earlier proposed that glandular trichomes might contribute to the high salt tolerance by excreting toxic ions (Gucci et al., 1997; Aguirre-Medina et al., 2002). The anatomical modifications in response to 200 μ M CuSO₄ was almost similar to that of the plantlets grown in 400 mM NaCl. The higher density of glandular trichomes in response to Cu stress was also found in *V. inermis*. Zehra et al. (2020) reported that, Cu stress amelioration studies using ABA in *Artemisia annua* exhibited similar observation. They concluded that, the increased glandular trichome density have a relation with artemisinin content in plants of arid parts.

The roots exhibited damages higher than leaves because of the direct contact with NaCl/CuSO₄. The epidermis and the arrangement of the aerenchymatous cortex cells was distorted in response to both the stresses (400 mM NaCl and 200 μ M CuSO₄). This was reported in plants like *B. cylindrica* in response to 0.25 mM CuSO₄ (Sruthi and Puthur, 2019). The entire cortical region in the plant *Chromolaena odorata* was found to be altered in studies with other heavy metals such as Hg and Fe (Swapna et al., 2015). Authors of these studies concluded that growth in hydroponic nutrient medium caused the cortical cell injury, which was caused by direct exposure of the metal to the soft root tissues.

The structural difference in the vasculature of both leaves and roots on exposure to NaCl is a sign of plantlets tolerance potential. The vascular cell thickness was much greater than control plantlets of *V. inermis* on being subjected to 400 mM NaCl. Salinity stress has been associated with increased lignin deposits in vascular tissues. The increased lignin deposits were observed in *V. inermis* subjected to the phloroglucinol-HCl test (Sarath et al.,

2024). Salinity causes stronger lignifications in the vascular tissue of *V. inermis*, which has been hypothesized to be a factor that restricts root growth and, as a result, constitutes a defense strategy against salt stress (Dolatabadian et al., 2011). In the case of Cu stress, *V. inermis* also exhibits higher amount of lignin as compared with control plantlets. The lignification in response to abiotic stresses, significantly contributes to the tolerance and in the case heavy metals, it helps in the sequestration of toxic metal in the cell wall. Lignin is one of the extra cell wall thickening material, which specifically provide rigidity and hydrophobicity. Besides this, the carboxyl and hydroxyl groups of the lignin tightly bind the Cu ions (Tugbaeva et al., 2022). This higher lignified cells in the root especially in the xylem walls validates the Cu phytostabilisation potential of *V. inermis*.

Saadeddin and Doddema (1986) reported that plantlets developing in low-water potential habitats frequently exhibit lignification in the metaxylem. According to Junghans et al. (2006), the conduit wall reinforcement *via* increased xylem wall thickness was more important than the xylem diameter in plants subjected to stress. The xylem with increased diameter collapses easily under stress conditions due to the lesser thickness. The thickened vessel walls and increased vessel density imparts more life and protect the vessels from stress-imposed implosion, and this safeguards the water-conducting xylem vessels under stress (Sarath et al., 2024).

5.12 Secondary metabolites

There is a close relationship between the accumulation of secondary metabolites and a plant's ability to withstand stress. When toxic heavy metal concentrations are over the threshold, they act as stressors and can cause changes in their secondary metabolism. The induction of secondary metabolite formation involves an array of cellular and molecular interactions and signalling pathways. In this study, phenols, terpenoids, alkaloids,

flavonoids, and glycosides are the major classes of compounds identified through HRLC-MS analysis in the leaves and roots of *V. inermis* treated with NaCl and CuSO₄. Phenolic compounds play a key role in regulating growth, reproduction, physiological properties, antioxidant as well as antimicrobial activities (Balasundram et al., 2006). They contain hydroxyl (-OH) and carboxyl (-COOH) groups, having the property to bind heavy metals. The chelating ability of these compounds can be related to the high nucleophilic nature of their aromatic rings. Previous studies have reported that abiotic stresses can up-regulate the expression of genes that encode key enzymes in the phenylpropanoid pathway to facilitate the accumulation of phenolic acids, which function as antioxidants and enable plants to acclimatise to abiotic stress (Isah, 2019). For example, the highest contents of polyphenolic compounds such as chlorogenic acid and isochlorogenic acid A were reported in *Lonicera japonica* under NaCl stress and the transcriptional activation of genes involved in the biosynthesis of these compounds were significantly increased under NaCl stress.

In *V. inermis*, despite biomass reduction, absolute amounts of vanilglycol and chlorogenic acid content was significantly increased by CuSO₄ treatment. This revealed that chlorogenic acid accumulation in leaves was the result of stimulation in synthesis of this compound under heavy metal stress. Furthermore, increased concentrations of sulochrin and oroxindin were observed in the CuSO₄-treated leaves. Sulochrin is a carboxylic ester and a member of benzophenone. These compounds can inhibit lipid peroxidation by trapping the alkoxyl radicals (Michalak, 2006).

Saponins, belonging to the class glucosinolates, play a key role in the rapid response and defense mechanisms of plants. Notoginsenoside A is a saponin that was detected in the NaCl-treated leaves, but no saponins were observed in both the control and CuSO₄-treated leaves. Application of 80–

140 mg/kg arsenic resulted in a significant increase in the total saponin content and squalene synthase activity in the rhizomes of plants. Martínez et al. (2014) reported that increased synthesis of glucosinolates having the capacity to act as osmoprotective compounds and maintain the leaf water balance in plants subjected to salt stress. Similar studies were reported for *Panax notoginseng* treated with varying concentrations of arsenic. Momilactone B is the common terpenoid compound found in control and NaCl-treated leaves. Momilactones A (MA) and B (MB) were reported to contribute to rice resistance against salt, drought, weed, UV, and heavy metal stresses (Okada et al., 2007). This indicates its confirmed role in stress tolerance.

As plant roots are in close contact with polluted soil, they may be more susceptible to changes in environmental factors such as heavy metal concentrations. Therefore, when exposed to environmental stress, plant roots will release a range of chemical compounds that may have an impact on the dynamics of soil nutrients and microbial activity in the rhizosphere. When compared to the control root, the number of phytochemicals was very high in both NaCl and CuSO₄-treated roots. A total of 42 compounds were resolved in the NaCl-treated root extract, whereas about 31 different phytochemicals were observed in CuSO₄-treated roots. During NaCl and Cu stress, it was noticed that the presence of phenolics was more pronounced in the root than the shoot. This could be the result of the fact that roots come in direct contact with metal and salt ions first and that stress-mediated effects can have tissue-specific effects.

According to HPLC studies by Dubey et al. (2018), phenolic acids such as p-hydroxybenzoic acid, vanillic acid, protocatechuic acid, caffeic acid, and p-coumaric acid were present in significant concentrations in Cr(VI)-stressed plant extracts. The presence of gallic acid was also found to

be at its highest concentration, confirming its crucial defence function in Cr(VI)-induced stress in plants. Gallic acid, ferulic acid, caffeic acid, oolonghomobisflavan A, and paradol are the phenolic compounds found in NaCl-treated roots of *V. inermis*. Caffeic acid, a metabolite of the phenylpropanoid pathway, can alter the growth, photosynthetic processes, and ROS production in plants. Chlorogenic acids (CGAs) belong to the ester group of phytochemicals that are produced when quinic acids and derivatives of cinnamic acids are combined. In response to both biotic and abiotic stresses, CGAs have been found to be synthesised through the shikimate and phenylpropanoid pathways (Ncube et al., 2014). The most prevalent hydroxyl cinnamic acid in plants, ferulic acid, exhibits antioxidant property by donating one hydrogen atom from its phenolic hydroxyl group in response to free radicals. Ferulic acid demonstrates a wide range of biological properties, including metal chelation, antioxidation, and enzyme activity modulation.

Additionally, various other phytoconstituents were found in *V. inermis* in response to NaCl and CuSO₄ and have the potential to treat a variety of human ailments. Therefore, the concurrent increase of phenolics and other compounds in plants holds promise for medical and pharmacological uses. At present no reports exist on the significance of these phytocompounds. In future detailed research is required to elucidate the role and importance of these phytochemicals.

The augmented production of secondary metabolites in plantlets exposed to NaCl and CuSO₄ was due to the higher phenylalanine ammonia-lyase activity. It was clearly corroborating with our results. The plantlets exposed to 400 mM NaCl and 200 μ M CuSO₄ showed higher *pal* expression as compared with control plantlets. Phenylalanine ammonia-lyase is the key regulatory enzyme for the synthesis of a large number of natural

compounds such as lignin, flavonoids, furanocoumarin, hydroxycinnamic acid esters etc. This enzyme particularly deaminate phenylalanine to yield trans cinnamic acid and ammonium and activate further phenylpropanoid pathway and produce different phenolic secondary metabolites (Cramer et al., 1989; Zhang and Liu, 2015). These metabolites are synthesised normally at a lower level and can be induced to a large extent in response to stress condition. The exposure of *Matricaria chamomilla* to 60 and 120 μM CuSO_4 results in higher *pal* activity than the control. The higher level of stress tolerance exhibited by this plant was largely due to the phenolic compounds and that was elicited by Cu through PAL enzyme (Kováčik and Bačkor, 2007).

5.13 Phytochelatins synthase and metal chelation

Phytochelatins (PCs) are thiol peptides that chelate heavy metals in plants. Phytochelatins synthase (PCS) produces PCs non-translationally from glutathione (Seregin and Kozhevnikova, 2023). The post-translational activation of PCS, a constitutively expressed enzyme, is well known to occur in the presence of heavy metals. The PCs have a general formula $(\gamma\text{-Glu-Cys})_n\text{-X}$ structure where $n = 2\text{--}11$, and X is often glycine and has better binding capacity with toxic metals. The increased expression of PCS in plantlets exposed to heavy metals is an intrinsic tolerance mechanism against toxic metals (Su et al., 2020; Liu et al., 2021). The enhanced expression of *pcs* was found in the plantlets of *V. inermis*. The plantlets treated with 400 mM NaCl and 200 μM CuSO_4 exhibited an enhancement in *pcs* expression as compared with the control plantlets. Similar enhancement of PCs was found in the roots of *Solanum nigrum* plants exposed to 200 $\mu\text{mol/L}$ Cu, contributing to a higher Cu accumulation in these organs, suggesting a role of PCs in the detoxification of this metal (Fidalgo et al., 2013). Cevher-Keskin et al. (2019) showed that upregulation of PCS was

observed in *Brassica nigra* plants subjected to 200 and 500 μM Cu in the growth medium. The role of PCs in salt stress tolerance was also reported. Beside the metal tolerance, PCs have role in NaCl stress. The plantlets of *A. thaliana* under salt stress over express *AtPCS2* and its mechanism against NaCl tolerance is not yet clear (Kim et al., 2019). They also conclude that, *AtPCS2* have role in proline biosynthesis and Na sequestration in *A. thaliana*. These findings imply that *AtPCS2* mediates osmotic adjustments and ion homeostasis through proline accumulation, hence protecting cell vitality preferentially against higher NaCl concentrations (Kim et al., 2019). These observations corroborate with the current study. The plantlets of *V. inermis* exhibits higher tolerance upto 400 mM NaCl and it is largely due to the higher level of PCs and it was clear from the higher expression of PCS. The increased glutathione content in the root and leaves of *V. inermis* may also contributes to PCs synthesis.

5.14 Exploring the bioprospecting potential of *V. inermis* following phytoremediation

Volkameria inermis, holds potential for bioprospecting, particularly after post phytoremediation. Restoring saline or copper-affected areas with *V. inermis* can also provide opportunities to harness the medicinal properties of the plant. The HR-LCMS analysis of *V. inermis* in response to NaCl and CuSO_4 elicit many compounds with pharmacological importance. Absolute amounts of vanlyglycol and chlorogenic acid content was significantly increased by CuSO_4 treatment. Notoginsenoside A is a saponin that was detected in the NaCl-treated leaves, but no saponins were observed in both the control and CuSO_4 -treated leaves. Additionally, various other phytoconstituents were found in *V. inermis* in response to NaCl and CuSO_4 . The concurrent increase of phenolics and other compounds in *V. inermis* holds promise for its use in pharmacology. At present no reports exist on the

significance of the phytochemicals detected in *V. inermis* in response to *NaCl* and *CuSO₄*. Detailed research is required to elucidate the role and importance of these phytochemicals.

The direct use of *Volkameria inermis* biomass in traditional Ayurvedic medicine is not advisable due to the accumulation of higher levels of toxic metals in the plant. Additionally, the concentration of active compounds in raw plants can fluctuate significantly based on environmental conditions, resulting in inconsistent therapeutic effects. A proper solution to this issue is to isolate the specific compound of interest. The isolation of contaminant-free metabolites from plants used for phytoremediation by using hydro-distillation and supercritical CO₂ extraction could have application in pharmaceuticals, cosmetics, or herbal products.

6. SUMMARY AND CONCLUSIONS

Physio-dynamism related to phytodesalination and phytoremediation of copper in *V. inermis* was evaluated under hydroponic culture conditions. Hydroponic culture provides a controlled growth condition with known concentration of constituents. Healthy stem cuttings of *V. inermis* were collected from natural habitats, and the cuttings were surface-sterilized. The surface sterilized stem cuttings were treated with 15 μM indole butyric acid (IBA) for root initiation. They were kept in paper cups (9×7 cm) filled with soil collected from the mangrove habitats for proper growth. After 80d of growth, the rooted cuttings were transferred to 300 ml half-strength modified Hoagland medium. The healthy plantlets of *V. inermis* were then subjected to varying concentrations of NaCl (0, 100, 200, 300, 400 and 500 mM) and CuSO₄ (0, 100, 150, 200 and 250 μM) for 20d under hydroponic culture conditions.

Based on the preliminary phase of the study, it was evident that *V. inermis* could effectively tolerate up to 400 mM of NaCl and 200 μM CuSO₄, and signs of severe stress effects were shown in plantlets grown in concentration above it. So, the detailed study of physio-dynamism related with phytoremediation and phytodesalination in *V. inermis* was carried out using 100 and 200 μM CuSO₄ and 100, 200, 300 and 400 mM NaCl.

The following is the summary of the most significant results from the current investigation:

- The growth of the plantlets under different concentrations of NaCl and CuSO₄ was analyzed using various plant growth parameters such as fresh and dry weight, leaf area, tissue water content and tolerance index. The fresh and dry weight of the leaves and roots of NaCl-treated plantlets declined proportionally with increasing NaCl

concentration, except for those treated with 100 mM NaCl. The promotion of plantlet growth by a modest concentration of NaCl (100 mM) compared to control plantlets confirms the plantlets' halophytic nature. The growth of plantlets under CuSO₄ treatment experienced the same condition with higher fresh and dry weight reduction only in higher concentrations of CuSO₄, wherein plantlets grown in the lower CuSO₄ concentration (100 µM) did not affect the fresh and dry weight. Lower CuSO₄ concentrations promote plant growth because Cu is known to positively influence many important processes such as photosynthesis, chlorophyll and lignin synthesis, acting as cofactor of enzymes, antioxidation mechanism, and stress mitigation.

- The improvement in the leaf area of *V. inermis* treated with a lower concentration of NaCl (100 mM) clearly indicates the beneficial effect of NaCl lower concentration. But at the same time, plant growth in other higher concentrations of NaCl (200, 300 and 400 mM) and 200 µM CuSO₄ had leaves with reduced leaf area in comparison with the control. The reduction in the expansion of leaves was due to the reduced cell division under severe stress, ultimately causing the reduction in the total dry mass of the plants.
- The efficacy of the plantlets under NaCl/metal stress was effectively measured by TI%. TI of leaves was reduced to 50% in 200 µM CuSO₄ treatment compared to the control plants. The TI of *V. inermis* was increased when exposed to 100 and 200 mM NaCl, but it declined when subjected to higher NaCl concentrations (300 and 400 mM). The biomass of the plantlets decreased as a result of reduced photosynthesis under stress, which had a negative impact on TI. The limited growth under NaCl/CuSO₄ stress was due to the utilization

of the energy for energizing tolerance mechanisms, and it was reflected in reduced fresh and dry weight.

- The tissue water content of the leaves and roots of plantlets were reduced compared to the respective control plantlets. The maximum reduction was found in plantlets grown in higher NaCl concentrations (400 mM). The hyper absorption of Na^+ and Cl^- reduces cellular water potential in NaCl-stressed plants. Like NaCl, higher concentrations of CuSO_4 negatively affect the tissue water content. The heavy metal-induced impairment in plant water relationship is due to stress-induced regulation of root hair formation. It reduces surface absorption, bio-membrane permeability and xylem differentiation, and these features directly affect the tissue water status.
- The stress imparted by different concentrations of NaCl and CuSO_4 was evaluated using stress intensity factors such as H_2O_2 , $\text{O}_2^{\bullet-}$, MSI, electrolyte leakage and lipid peroxidation. The exposure of *V. inermis* to NaCl and CuSO_4 treatments causes oxidative stress by the upregulation of the ROS such as H_2O_2 and $\text{O}_2^{\bullet-}$ due to the damage of the photosystems and impaired thylakoid electron transport process. Moreover, the unregulated electron flow due to the augmented oxidation of metabolites in cell organelles, including chloroplast, peroxisomes and mitochondria in NaCl and CuSO_4 -treated plants, causes a rapid increase in ROS production. The production of H_2O_2 and $\text{O}_2^{\bullet-}$ was higher in higher concentrations than the plantlets grown in the lower concentrations of NaCl and CuSO_4 . The *V. inermis* plantlets exposed to lower concentrations of NaCl and CuSO_4 have lesser accumulation of ROS and therefore lesser amounts of MDA, indicating lower lipid peroxidation. The ROS generation in response

to NaCl and CuSO₄ resulted in the peroxidation of lipids in membranes. A high concentration of ROS in the cellular environment, harms the structure of cells and biomolecules by damaging DNA, proteins, and lipids in cell membranes. As roots are the first and over-exposed plant part, the MDA content formed in the roots is much higher than in the leaves and therefore damage to the roots are much higher. This can severely affect root growth compared to leaves.

- The photosynthetic efficacy of *V. inermis* was modulated in response to NaCl and CuSO₄, and it was assessed using different photosynthetic parameters such as chlorophyll and carotenoid content, CSI, Photosystems (PSI and PSII) activities, chlorophyll *a* fluorescence and gas exchange parameters. The total chlorophyll content in the leaves of the plantlets decreased with increasing concentrations of NaCl, except for the plantlets subjected to 100 mM NaCl. Similarly, lower concentrations of CuSO₄ positively influenced and enhanced the total chlorophyll content over the control. This indicates that the plant is tolerant to low concentrations of Cu and this metal has a specific role in plant growth and development. The decreased chlorophyll content in plantlets exposed to higher CuSO₄ concentration could be due to the obstruction of enzymes involved in the biosynthesis of chlorophyll, and the higher Cu in the growing habitat competes with Mg and Fe, leading to the decreased uptake of the latter. Moreover, the intermediate product in the formation of chlorophyll, protochlorophyllide, is inhibited by higher Cu concentrations. The higher Cu in plant body potentially bind with the -SH groups of the aminolevulinic acids (ALA) dehydratase and protochlorophyllide reductase and thereby reduces its enzymatic capacity and inhibits chlorophyll biosynthesis.

- The carotenoid content of *V. inermis* increased in response to NaCl and CuSO₄ treatments. The increased carotenoid content reduces the triplet state of chlorophyll, thereby eliminating the formation of singlet oxygen, and is responsible for the protection of photosynthetic machinery. Moreover, enhanced carotenoid content can be an adaptive strategy to protect plants from stress-induced injury.
- The photosystem activities (PSI and PSII) are sensitive to NaCl and CuSO₄. PSI activity was found to be reduced under both NaCl and CuSO₄ stress in *V. inermis* plantlets. The higher accumulation of Na/Cu in the plant parts causes damage to the thylakoid membrane and thereby reduces the photosystem activities. The higher NaCl and CuSO₄ treatments cause degradation of the thylakoid protein complex and destabilize the thylakoid membrane, reducing photosystem activities. As a result of Cu stress, the damaged antenna molecules and reaction centres inactivate the PSII donor side, inhibiting PSII electron transfer and thereby reducing PS II activity.
- Chlorophyll *a* fluorescence is a non-destructive, efficient, and quick method for analyzing light-dependent photosynthetic processes in plants. Chlorophyll *a* fluorescence transient OJIP curves measured in *V. inermis* plantlets treated with lower concentrations of NaCl or CuSO₄ were not significantly impacted. However, compared to the control plantlets, those subjected to higher NaCl and CuSO₄ concentrations exhibited OJIP curve with dramatic changes. The reduction in F₀ in *V. inermis* grown under higher NaCl and CuSO₄ indicates the vulnerability of the plant towards Na and Cu stresses. The decreased F_m under stressful circumstances denotes the dissociation of the PSII light-harvesting complexes, and it may also be due to the decrease of the pigment content in plantlets grown under

higher NaCl and CuSO₄ concentrations. The stress-induced destabilization of PSII and inactivation of D1 proteins severely affect the light capture and quantum yield. The membrane damages associated with 400 mM NaCl and 200 µM CuSO₄ treatments in *V. inermis* reduce the maximum quantum yield of PSII (Fv/Fm) and maximum efficiency of the water splitting complex (Fv/Fo). The reduction of PI_{ABS} and PI_{total} in response to higher NaCl and CuSO₄ concentrations and the increase of the same parameters in lower NaCl and CuSO₄ concentrations specifically validate the stress effects of higher Na and Cu concentration in the growth of *V. inermis*.

- The phenomenological energy pipeline models indicate the flow of energy and the related photochemistry of *V. inermis*. The phytotoxicity of the NaCl and CuSO₄ increased with the increasing concentration and accordingly caused the reduction in the ABS/CSm, TRo/CSm and ETo/CSm. The reduction in the functional reaction centres leads to reduced photosynthetic efficiency. The decrease in energy absorption by chlorophyll molecules is due to a drop in PSII antenna size or a structural change in minor antenna LHC components. The enhancement in DIo/CSm of plantlets of *V. inermis* was due to the ineffectiveness of the reaction centres in effectively utilizing the absorbed light energy for electron transport. The increase of inactive reaction centres and the reduced electron transport due to the reduction of the functional Q_A pool causes an increment in DIo/CSm.
- The physiological modulations in osmolytes play a critical role in stress mitigation. The osmolality of the NaCl and CuSO₄ stressed plants was analyzed, and the accumulation of osmolytes in terms of total soluble sugar, total soluble proteins, total free amino acids and

proline content were also determined. As part of NaCl tolerance, the toxic Na^+ is sequestered into the vacuole, resulting in the solute potential difference between the vacuole and the cytoplasm. This conflict in the solute potential can be neutralized by the accumulation of osmolytes in the cytoplasm. The NaCl-treated plantlets showed a higher accumulation of sugars, proline, amino acids and proteins, which can function as compatible solutes. The enhanced accumulation of osmoprotectants safeguards the organelles, bio-membrane, and cytosolic enzymes, and it has been demonstrated that the accumulation of compatible solutes during stress conditions inhibits plant growth as the photosynthate is diversified towards the synthesis of compatible solutes.

- On exposure to NaCl and CuSO_4 , the proline content gradually increased in the leaves and roots. In comparison to the roots, the increase was greater in the leaves. Proline has a significant capacity for hydration. Its hydrophilic part can bind to water molecules, whereas its hydrophobic terminus can bind to proteins. Proteins containing proline can bind more water, avoiding dehydration and denaturation in response to osmotic stress. Higher proline content is essential for osmoregulation, ROS abatement, and the stabilization of cellular components. In addition to acting as osmoprotectants, the increase in proline and total amino acids concentration also serves as a source of nitrogen and carbon. Consequently, it regulates nitrogen availability under high-stress conditions and during the recovery from stress. Proline also plays key roles in redox buffering, acting as molecular chaperones and maintaining the integrity of membranes and proteins.

- In *V. inermis* plantlets, the counteraction against oxidative stress due to NaCl and CuSO₄ treatments was by two major mechanisms, which include enzymatic and non-enzymatic antioxidants. The non-enzymatic antioxidants include ascorbate, glutathione, phenolics and flavonoids and enzymatic antioxidants such as SOD, POD and APX. It reduces or inhibits the cellular damage caused by ROS in *V. inermis* plantlets. The present investigation also showed increased activity of the enzymes SOD, POD, and APX when plantlets were exposed to NaCl and also observed that the activity of these enzymes were higher in plantlets subjected to 200 µM CuSO₄ as compared with the control plantlets. The increment in the activities of antioxidant enzymes in the plantlets exposed to higher concentrations of NaCl/CuSO₄ and lesser activities in plantlets treated with lower concentrations clearly correlated with the ROS generation in *V. inermis* and also indicates that the ROS generated acted as induction signal for the activation of the anti-oxidation process. The increment in the enzymatic antioxidants in *V. inermis* under NaCl and CuSO₄ stresses helped them to cope with these severe situations and thereby showed higher tolerance against NaCl and CuSO₄ stresses.
- Increased synthesis of total phenolics, ascorbate, glutathione and flavonoids were found in *V. inermis* in response to NaCl and CuSO₄ stress. It attributes the tolerance potential of *V. inermis* plantlets towards both these stresses. In the case of Cu stress (redox active metal), the highly augmented phenolic compounds act as pro-oxidants and participate in H₂O₂ scavenging. The increase of phenolics content attributes metal chelating efficiency to plantlets and helps in the formation of metal-phenolics complex in the plant biomass. The glutathione content in leaves and roots was also increased with increasing concentrations of NaCl and CuSO₄. The

main source of nonprotein thiol in most plant cells is glutathione. The thiol group's nucleophilic character is crucial for forming mercaptide bonds with metals and reacting with particular electrophiles. The higher synthesis of glutathione may also help to synthesise PCs, which have the capacity to chelate and detoxify the metals. A higher level of ascorbate accumulated in *V. inermis* in response to NaCl and CuSO₄ treatments, and there was a progressive increase in ascorbate content up to 300 mM NaCl concentration; further, it gets reduced. But the reduction was not lesser than that of the control and was maintained as like that of control plantlets. The reduction in higher concentration (400 mM NaCl) could be due to irreparable damages in the ascorbate biosynthesis pathway due to the toxicity imparted by High NaCl concentration. The accumulation of flavonoids with the highest antioxidant capacity was positively correlated with an upsurge in ROS concentration brought on by stress. The NaCl/CuSO₄ exposure positively influenced the flavonoid biosynthesis, and the increase was maximum in higher concentration treatments of NaCl and CuSO₄.

- The phytoremediation potential of *V. inermis* was evaluated by assessing the bioaccumulation of Na and Cu in different plant parts. The bioaccumulation of Na and Cu in *V. inermis* plantlets increased gradually with the concentration of NaCl and CuSO₄ in the medium. Among the different plant parts of the plantlets exposed to NaCl, the roots showed a higher accumulation percentage of Na. Similar observation was also found in the case of Cu, wherein maximum accumulation was found in root tissues. Next to roots, higher rate of Cu accumulation was observed in the stem of *V. inermis*. The efficiency of *V. inermis* for phytoremediation and phytodesalination

was amply demonstrated by the increased concentration of Na and Cu in its plant parts.

- The TF decreased in plantlets exposed to NaCl, and maximum reduction was recorded in plantlets exposed to 300 and 400 mM NaCl. The TF of the plantlets treated with 200 μ M CuSO₄ was very low and even below 1 (0.096 ± 0.004). Another interesting factor was that the treatment with 100 μ M CuSO₄ resulted in higher TF (1.59 ± 0.079), and it was higher than that of control plantlets. Stabilizing Cu in the root tissues due to lower translocation to shoot helps the plants to tolerate severe Cu stress in leaves, thereby protecting photosynthetic machinery. The sequestration of Cu in the root cell wall and other parts contributes to the phytostabilization ability of *V. inermis*. The Cu ions uptake were increasingly complexed with the cell wall, as evidenced by the results of subcellular localization of metal ions. The highest accumulation was in the root tissue's cell wall and wall debris. The stabilization of Cu in the root and low TF, particularly due to the cell wall sequestration, was clearly evident from subcellular studies.
- The subcellular localization studies in *V. inermis* revealed the localization of the Na, Cu, K and Ca in different cell fractions. The detoxification of excess Na⁺ in the NaCl-treated *V. inermis* was facilitated to a greater extent through the vacuolar sequestration without causing much deleterious effect on other active parts of the cell. In addition, it was also noted that the concentration of Ca increased in fraction 4 (ribosomes, vacuoles, and cytoplasm), demonstrating the inseparable role of Ca in helping in the vacuolar sequestration of excess Na⁺ and thereby imparting tolerance to NaCl.

- The uptake of Na was correlated with the increased level of K in the fraction containing ribosomes, vacuoles, and cytoplasm, which could be due to the structural similarity of both ions, and K effectively counters NaCl-induced stress by mediating various enzymes' activities.
- Salt stress-induced variation in mineral content may trigger various ionic imbalance in plants. In the current study, the increased accumulation of Na negatively affects the uptake rate of other ions, such as Ca and Fe, and the concentration of macronutrients (K, Mg, Ca) tends to have drastic variations in all the tissues, i.e., leaf, stem and root subjected to CuSO₄. NaCl and CuSO₄ treated plantlets have limited carbon flux, which might be due to the diminished rate of photosynthesis, limited stomatal conductance, and restricted uptake of CO₂ under stressed conditions.
- The cellular-level structural alterations were evaluated using anatomical studies. There was no drastic characteristic changes or damages in the leaf anatomy of *V. inermis* subjected to 400 mM NaCl. This clearly indicates its tolerance and maintenance of cellular integrity under NaCl stress. The plantlets exposed to NaCl and CuSO₄ had more glandular trichomes, which might contribute to the tolerance by adding toxic ions to excretion. The roots exhibited damages higher than leaves because of the direct contact with NaCl/CuSO₄. The epidermis and the arrangement of the aerenchymatous cortex cells was distorted in response to both the stresses.
- NaCl causes stronger lignifications in the vascular tissue of *V. inermis*, which has been hypothesized to be a factor that restricts root growth and, as a result, constitutes a defense strategy against salt stress. In

response to Cu stress, the lignification significantly contributes to tolerance and helps to sequester toxic metals in the cell wall. The carboxyl and hydroxyl groups of the lignin tightly bind the Cu ions. Moreover, the higher lignin content specifically provides rigidity and hydrophobicity. These higher lignified cells in the root, especially in the xylem walls, validate the Cu phytostabilization potential of *V. inermis*.

- The secondary metabolites accumulated in *V. inermis* in response to NaCl and CuSO₄ were determined by HR LC-MS. The methanolic extract of control leaves showed a total of 29 compounds identified at different retention times. Furthermore, the highest number of non-volatile compounds were observed in CuSO₄-treated leaves (43 compounds), followed by the control leaves (29 compounds) as well as NaCl-treated leaves (18 compounds). Notoginsenoside A is a saponin detected in the NaCl-treated leaves; no saponins were observed in either the control or CuSO₄-treated leaves. Barbinine is an alkaloid compound found in NaCl-treated leaves, whereas bebeerine has been the common alkaloid compound detected in the control and CuSO₄-treated leaves. Similarly, momilactone B is the common terpenoid compound found in control and NaCl-treated leaves, and (+)-sophorol is the flavonoid compound observed in both control and CuSO₄-treated leaves. The synthesis of Chlorogenic acid in leaves was stimulated under Cu treatment. Sulochrin is a carboxylic ester and a member of benzophenone that inhibits lipid peroxidation by trapping the alkoxyl radicals.
- Twenty-eight non-volatile phytocompounds were identified in the control root at different retention times. Compared to the control root extract, the number of phytocompounds was very high in NaCl and

CuSO₄-treated roots. A total of 41 compounds were resolved in the NaCl-treated root extract, whereas about 31 different phytochemicals were observed in CuSO₄-treated roots. Gallic acid, ferulic acid, caffeic acid, oolonghomobisflavan A, and paradol are the phenolic compounds found in NaCl-treated roots of *V. inermis*. Caffeic acid, a metabolite of the phenylpropanoid pathway, has the potential to alter the growth, photosynthetic processes, and ROS production in plants. Ferulic acid demonstrates many biological properties, including metal chelation, antioxidation, and enzyme activity modulation.

- The higher phenylalanine ammonia-lyase activity showed the augmented production of secondary metabolites in plantlets exposed to NaCl and CuSO₄. RT-qPCR evaluated the gene expression of *pal*. The plantlets exposed to 400 mM NaCl and 200 μM CuSO₄ showed higher *pal* expression than control plantlets. This enzyme deaminates phenylalanine to yield trans-cinnamic acid and ammonium, further activating the phenylpropanoid pathway and producing different phenolic secondary metabolites. The higher phenolic compounds provide enhanced antioxidant potential and also play a role in metal chelation during metal stress.
- The plantlets treated with 400 mM NaCl and 200 μM CuSO₄ had enhanced *pcs* expression compared to the control plantlets. Phytochelatins synthase (PCS) produces PCs non-translationally from glutathione. The increased expression of *pcs* in plantlets exposed to NaCl and heavy metals is an intrinsic tolerance mechanism. These thiol peptides help the plant to chelate heavy metals.

As per the findings, *V. inermis* withstand higher concentrations of NaCl and CuSO₄ in growing habitats by modulating their physio-anatomical

features. The dynamism of antioxidative machinery under high NaCl and CuSO₄ concentrations effectively safeguard the plantlets under oxidative stress. The higher ROS burst in the plantlets grown under higher concentrations of NaCl and CuSO₄ acts as a signal for progressive cascades of antioxidant machinery, leading to the effective scavenging of ROS and thereby reducing its negative impacts. The conflict in the solute potential due to higher concentration of NaCl and CuSO₄ can be neutralized by the accumulating osmolytes (sugars, proline, amino acids and proteins) in the cytoplasm. The accumulation of osmolytes maintain the osmotic potential of *V. inermis* under stress. Stress-induced lignification of cells, particularly in the vascular region, aids in their survival and protects the vascular tissue from implosion. The thicker wall of the root xylem efficiently sequesters Na and Cu, preventing further translocation and enhancing their phytostabilizing properties. The impacts of the stress were ameliorated by modulating their active secondary metabolites production and it was clear from higher phenylalanine ammonia-lyase activity in plantlets exposed to NaCl and CuSO₄. From these, *V. inermis* can suggested to be a good option for phytodesalination and copper phytoremediation in salt and copper affected areas since it is equipped with strong NaCl/ CuSO₄ tolerance strategies.

RECOMMENDATIONS

The physio-dynamism related to phytodesalination and copper phytoremediation in *V. inermis* was studied in detail. The present study proposes the following recommendations for the future research.

- To conduct transcriptomics and proteomics investigations in *V. inermis* as they will offer a significant understanding of the molecular mechanisms behind plants responses to NaCl and CuSO₄ stresses.
- Identification, isolation, and characterisation of salt and copper-tolerant genes in *V. inermis* will give a new way to manipulate plants so as to develop tolerance towards NaCl and CuSO₄.
- The field trials with *V. inermis* in saline-affected and copper-contaminated lands should be carried out, which could be a feasibility study for the ecological restoration of coastal and copper-contaminated lands.
- Studies should be focused on the area of NaCl and CuSO₄-induced secondary metabolite production in *V. inermis* and its utility for medical purposes considering the already known information on its medical importance.
- Afforestation and ecological restoration of polluted coastal areas (salt and copper) with *V. inermis* gives an additional advantage of exploring its large-scale utilization for medicinal purpose.

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PUBLICATIONS AND PRESENTATIONS

Articles

1. **Sarath, N. G.**, Shackira, A. M., and Puthur, J. T. (2024). Adaptive physio-anatomical modulations and ionomics of *Volkameria inermis* L. in response to NaCl. *International Journal of Phytoremediation*, 26(1), 114-130. <https://doi.org/10.1080/15226514.2023.2229443> (IF-3.7).
2. Sanjana, S., Jazeel, K., Janeeshma, E., **Nair, S. G.**, and Shackira, A. M. (2024). Synergistic interactions of assorted ameliorating agents to enhance the potential of heavy metal phytoremediation. *Stress Biology*, 4(1), 13. <https://doi.org/10.1007/s44154-024-00153-1>
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Papers Presented

1. **Sarath, N. G.** and Puthur, J. T. (2023). 'Understanding the functional mechanisms operated in *Volkameria inermis* L. exposed to copper' in the National seminar on 'Green Economy Concepts and Perspectives', conducted by Department of Botany, Govt. College, Madappally, Vadakara, Kozhikode, 26-27 October 2023.

2. **Sarath, N. G.** and Puthur, J. T. (2023). 'Volkameria inermis L., a potent candidate for the revitalization of salinity affected lands' in the National seminar on 'Plant Biodiversity, Genetics, Breeding and Biotechnology', conducted by Department of Botany, University of Calicut, 7-8 December 2023.
3. **Sarath, N. G.** and Puthur, J. T. (2022). 'Analysis of metal accumulation and phytochelatin synthase expression underpin Cu phytoremediation potential of *Volkameria inermis* L.' in the International Conference on 'Physiological and Molecular Mechanisms of Abiotic Stress Tolerance in Plants' jointly organized by University of Calicut and Indian Society for Plant Physiology (ISPP), October 26-28, 2022.
4. **Sarath, N. G.** and Puthur J. T. (2019). 'Photochemistry associated with phytodesalination potential of *Clerodendrum inerme* (L.) Gaertn.' in the International Conference on 'Exploring the Scope of Plant Genetic Resources', conducted by University of Kerala, May 22-24, 2019.
5. **Sarath, N. G.** and Puthur J. T. (2019). 'NaCl stress amelioration potential of *Clerodendrum inerme* (L.) Gaertn.: Role of antioxidants and osmo-protectants' in the National Conference of Plant Physiology (NCP-2019) on 'Plant Productivity and Stress Management', organized by Kerala Agricultural University, Thrissur, Kerala on December 19-21, 2019.
6. **Sarath, N. G.** and Puthur J. T. (2019). 'Ecophysiological studies in relation to heavy metal content in different strategic areas or plants of Kadalundi-Vallikkunnu community reserve' in the 31st Kerala Science Congress, Fatima Mata College, Kollam, February 2-3, 2019.

Best paper awards

- Received **Best paper award** in the International conference on 'Physiological and molecular mechanisms for abiotic stress tolerance in plants', jointly organized by University of Calicut and Indian Society for Plant Physiology (ISPP), October 2022.
- Received **Best paper award** in National seminar on "Plant Biodiversity, Genetics, Breeding and Biotechnology", conducted by Department of Botany, University of Calicut, 7-8 December 2023.
- Received **Best paper award** in the 33rd Kerala Science Congress on January 2021.