

**UNRAVELLING THE ADAPTIVE MECHANISMS OF
ALTERNANTHERA TENELLA COLLA TO HEAVY METAL
STRESS THROUGH PHYSIOLOGICAL, BIOCHEMICAL
AND MOLECULAR ANALYSES**

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

DOCTOR OF PHILOSOPHY IN BOTANY

By

FIRDOUS K. A.

Under the guidance of

Dr. Resmi M. S.



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Certificate

This is to certify that no corrections were recommended by the adjudicators in the thesis entitled **"UNRAVELLING THE ADAPTIVE MECHANISMS OF ALTERNANTHERA TENELLA COLLA TO HEAVY METAL STRESS THROUGH PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR ANALYSES"** submitted by **Firdous K. A.**, as per the adjudication report. It is also certified that the contents in the thesis and the CD submitted are one and the same.

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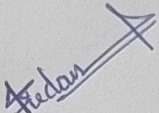
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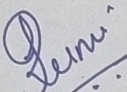
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Declaration

I, Firdous K.A., hereby declare that the work presented in the thesis entitled "Unravelling the adaptive mechanisms of *Alternanthera tenella* Colla to heavy metal stress through physiological, biochemical and molecular analyses" is based on the original work carried out by me under the guidance of Dr. Resmi M.S. 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 was found within the permissible limit. I also declare that the thesis is free from AI generated contents.

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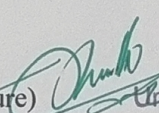
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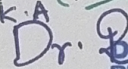


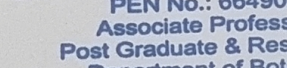
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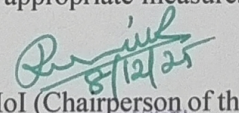
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ABSTRACT

Industrial development has resulted in widespread heavy metal contamination across ecosystems and pose serious risks to biodiversity, plant and human health. Despite this, the phytoremediation potential of riparian vegetation in industrial zones remains underexplored. The present study was conducted to identify a potential phytoremediator and to investigate its adaptive mechanisms in response to heavy metal stress. The study was conducted in three interconnected chapters, each addressing a specific aspect of heavy metal tolerance and detoxification.

The first chapter focused on the phytosociological assessment of riparian vegetation in the industrial zone of Kanjikode along Walayar Riverbank to identify potential phytoremediators of heavy metal contamination. A total of 97 species belonging to 32 families were recorded, with Poaceae and Asteraceae being the most dominant. Therophytes were prevalent indicating their ability to survive in disturbed environments. Among the flora, invasive species such as *Alternanthera tenella*, *Persicaria barbata* and *Croton bonplandianus* showed high abundance. *A. tenella* emerged as dominant based on its Importance Value Index (IVI), wide distribution and high efficiency to accumulate and translocate heavy metals like chromium and zinc, demonstrating its strong phytoremediation potential. These characteristics make it a promising candidate for phytoremediation in contaminated riparian areas.

The second chapter involved hydroponic studies to evaluate the morphological, anatomical, physiological and biochemical responses of *A. tenella* to cadmium (Cd), chromium (Cr) and lead (Pb) exposure. Rooted plants were treated with Cd (170 μ M), Cr (240 μ M) and Pb (680 μ M) in Hoagland nutrient medium. Morphological assessments revealed gradual reductions in root and shoot growth, leaf area and biomass under heavy metal stress. Cr caused relatively higher inhibitory effects compared to Cd and Pb. Anatomical observations showed adaptive modifications such as thickened xylem walls, reduced xylem diameter and deposition of electron dense granules in cortical and vascular tissues. These changes indicate metal

sequestration and restricted translocation of metals to aerial parts. Physiological analyses exhibited significant decline in photosynthetic pigments, stomatal conductance, and water content. Antioxidant enzymes and stress metabolites like proline and phenol were accumulated under heavy metal exposure, indicating activation of reactive oxygen species scavenging and osmotic adjustment. FTIR spectral shifts confirmed metal binding with functional groups such as hydroxyl, carboxyl, and amide groups in the plant tissues. A comparison of biomass, root-shoot distribution and metal accumulation potential indicated that *A. tenella* possesses high bioconcentration and bioaccumulation factors. This reflects the plant's selective accumulation of Cd and Pb in roots (phytostabilization) and efficient Cr translocation to shoots (phytoextraction).

Among the three heavy metals tested, chromium triggered the strongest transcriptional and metabolic changes in *A. tenella*. This led to a detailed transcriptomic and metabolomic profiling presented in chapter three. Metabolomic analysis showed significant accumulation of flavonoids, organic acids, amino acids, polyols, and fatty acids, which likely contribute to metal chelation, oxidative stress mitigation, and maintenance of redox balance. Transcriptomic profiling revealed the upregulation of genes associated with metal transport, chelation, antioxidant defence, and stress signalling and downregulation of photosynthetic and cell wall remodelling genes. RT-PCR validation of eight selected genes across leaves, stems, and roots supported these results. A strong induction of HIPP39, ABCC2, GST, YSL1, and PCS was observed in roots and leaves, while XTH, LAC6, and CASP were downregulated under prolonged Cr exposure. Together, these findings offer better understanding of the molecular and physiological basis of Cr tolerance in *A. tenella* and understanding of plant-metal interactions. The ability of *A. tenella* for chromium phytoextraction and its ecological dominance in polluted riparian habitats makes it a promising species for phytoremediation and ecosystem restoration.

Keywords: Amaranthaceae, detoxification, transcriptomics, metabolomics, phytosociology, riparian.

സംഗ്രഹം

വ്യാവസായിക വികസനം ആവാസവ്യവസ്ഥയിലുടനീളം വ്യാപകമായ ഘനലോഹ മലിനീകരണത്തിന് കാരണമായിട്ടുണ്ട്. ഇത് ജൈവവൈവിധ്യത്തിനും സസ്യജാലങ്ങൾക്കും മനുഷ്യന്റെ ആരോഗ്യത്തിനും ഗുരുതരമായ പ്രത്യാഘാതങ്ങൾ സൃഷ്ടിക്കുന്നു. എങ്കിലും, വ്യവസായ മേഖലകളോട് ചേർന്നുള്ള നദീതീര സസ്യങ്ങളുടെ ഫൈറ്റോറെമീഡിയേഷൻ സാധ്യതകൾ ഇതുവരെ പരിമിതമായി മാത്രമേ പര്യവേക്ഷണം ചെയ്യപ്പെട്ടിട്ടുള്ളൂ. ഈ സാഹചര്യത്തിൽ, ഒരു യോജിച്ച ഫൈറ്റോറെമീഡിയേറ്ററെ തിരിച്ചറിയുന്നതിനും ഘനലോഹ സമ്മർദ്ദത്തിനെതിരായുള്ള അതിന്റെ അനുകൂലന പ്രക്രിയകൾ മനസ്സിലാക്കുന്നതിനുമായി നടത്തിയ ഈ പഠനം മൂന്നു അധ്യായങ്ങളിലായി അവതരിപ്പിച്ചിരിക്കുന്നു.

വാളയാർ നദീതീരത്തുള്ള കഞ്ചിക്കോട് വ്യാവസായിക മേഖലയിലെ നദീതീര സസ്യജാലങ്ങളുടെ ഫൈറ്റോസോഷ്യോളജിക്കൽ വിലയിരുത്തലിലൂടെ ഘനലോഹ മലിനീകരണത്തെ ശുദ്ധീകരിക്കാൻ കഴിവുള്ള സസ്യങ്ങളെ കണ്ടെത്തുകയായിരുന്നു ലക്ഷ്യം. 32 കുടുംബങ്ങളിൽ നിന്നായി ആകെ 97 ഇനം രേഖപ്പെടുത്തിയവയിൽ പോയേസി, ആസ്റ്ററേസി തുടങ്ങിയ കുടുംബങ്ങളുടെ ആധിക്യം കണ്ടെത്തി. ശിഥിലമായ അന്തരീക്ഷത്തിൽ അതിജീവിക്കാനുള്ള കഴിവ് സൂചിപ്പിക്കുന്ന രീതിയിൽ തെറോഫൈറ്റുകൾ വ്യാപകമായിരുന്നു. ആൾട്ടർനന്തെറ ടെനെല്ല, പെർസിക്കേറിയ ബാർബറ, ക്രോട്ടൺ ബോൺപ്ലാൻഡിയനസ് തുടങ്ങിയ അധിനിവേശ സസ്യങ്ങൾ ഉയർന്ന സാന്നിധ്യം കാണിച്ചു. എ. ടെനെല്ലയുടെ ഉയർന്ന IVI മൂല്യം, വ്യാപകമായ വിതരണശേഷി, ക്രോമിയം, സിങ്ക് തുടങ്ങിയ ഘനലോഹങ്ങൾ സഞ്ചയിക്കുന്നതിനും സംവഹനം ചെയ്യുന്നതിനുമുള്ള ഉയർന്ന കാര്യക്ഷമത എന്നിവ അതിന്റെ ശക്തമായ ഫൈറ്റോറെമീഡിയേഷൻ സാധ്യത പ്രകടമാക്കുന്നു. മലിനമായ നദീതീര പ്രദേശങ്ങളിൽ ഫൈറ്റോറെമീഡിയേഷനുള്ള ഒരു പ്രതീക്ഷാജനകമായ സസ്യമായി ഈ സവിശേഷതകൾ ഇതിനെ മാറ്റുന്നു.

രണ്ടാം അധ്യായത്തിൽ, ഹൈഡ്രോപോണിക് രീതിയിൽ കൃഷി ചെയ്ത എ. ടെനെല്ല ചെടികളെ കാഡ്മിയം (Cd - 170 μ M), ക്രോമിയം (Cr - 240 μ M), ലെഡ് (Pb - 680 μ M) എന്നിവയുടെ ആഗിരണത്തിന് വിധേയമാക്കി രൂപഭേദ, ഘടന, ശാരീരികവും ജൈവരാസപരവുമായ പ്രതികരണങ്ങൾ വിലയിരുത്തി. ഹോസ്റ്റാൻഡ് പോഷക മാധ്യമത്തിൽ ഘനലോഹങ്ങളുടെ സമ്മർദ്ദത്തിൽ വളർന്ന ചെടികളിൽ വേരിന്റെയും തണ്ടിന്റെയും വളർച്ച, ഇലകളുടെ വിസ്തീർണ്ണം, ജൈവഭാരം എന്നിവയിൽ ക്രമേണ കുറവുണ്ടായതായി രൂപാന്തര വിലയിരുത്തലുകൾ വെളിപ്പെടുത്തി. Cr-നുമായ സ്വാധീനം Cd, Pb എന്നിവയെ അപേക്ഷിച്ച് കൂടുതൽ ശക്തമായിരുന്നു. കട്ടികൂടിയ സൈലം ഭിത്തികൾ, സൈലം വ്യാസം കുറയൽ, കോർട്ടിക്കൽ, സംവഹന കലകളിൽ ഇലക്ട്രോൺ സാന്ദ്രമായ ഗ്രാനൂൾ നിക്ഷേപം തുടങ്ങിയ ഘടനാപരമായ മാറ്റങ്ങൾ രേഖപ്പെടുത്തി. ഈ മാറ്റങ്ങൾ ലോഹങ്ങളുടെ വിഘടനത്തെയും മുകൾ ഭാഗങ്ങളിലേക്കുള്ള ലോഹങ്ങളുടെ പരിമിതമായ സംവഹനത്തെയും സൂചിപ്പിക്കുന്നു. ഫോട്ടോസിന്ററിക് വർണകങ്ങൾ, സ്റ്റോമറ്റൽ കണ്ടക്റ്റൻസ്, ജലത്തിന്റെ അളവ് എന്നിവയിൽ ഗണ്യമായ കുറവും പ്രോലിൻ, ഫിനോൾ തുടങ്ങിയ സ്കെസ് മെറ്റബോളൈറ്റുകളുടെ വർധനയും ശ്രദ്ധിക്കപ്പെട്ടു. ഇത് റിയാക്ടീവ്

ഓക്സിജൻ സ്പീഷീസുകൾ ശോഷണം ചെയ്യുന്നതിലും ഓക്സോട്ടിക് ക്രമീകരണത്തിലും സജീവമാക്കുന്നതിനെ സൂചിപ്പിക്കുന്നു. സസ്യകലകളിലെ ഹൈഡ്രോക്സിൽ, കാർബോക്സിൽ, അമൈഡ് ഗ്രൂപ്പുകൾ പോലുള്ള ഫങ്ഷണൽ ഗ്രൂപ്പുകളുമായി ലോഹബന്ധനം ഉണ്ടാകുന്നതായി FTIR സ്പെക്ട്രൽ ഷിഫ്റ്റുകൾ സ്ഥിരീകരിച്ചു. ജൈവഭാരം, വേർ-തണ്ട് വിതരണം, ലോഹ ശേഖരണ ശേഷി എന്നിവയുടെ താരതമ്യം എ. ടെനെല്ലയ്ക്ക് ഉയർന്ന ബയോകോൺസെൻട്രേഷനും ബയോഅക്യുമുലേഷൻ ഘടകങ്ങളും ഉണ്ടെന്ന് സൂചിപ്പിക്കുന്നു. Cd, Pb എന്നിവയുടെ സെലക്ടീവ് ശേഖരണവും (ഹൈറ്റോസ്റ്റാബിലൈസേഷൻ) Cr ഫലപ്രദമായി ഇലകളിലേക്ക് മാറ്റുന്നതിനിലൂടെ ഹൈറ്റോഎക്സ്‌ട്രാക്ഷനും സാധ്യമാകുന്നു.

പരിശോധിച്ച ഭാരലോഹങ്ങളിൽ ക്രോമിയമാണ് സസ്യങ്ങളിൽ ഏറ്റവും ശക്തമായ മാറ്റങ്ങൾക്ക് കാരണമായത്. ആയതിനാൽ Cr നൽകിയ എ. ടെനെല്ലയിലെ വിശദമായ ട്രാൻസ്ക്രിപ്റ്റോം, മെറ്റബോളിക് പ്രൊഫൈലിംഗ് പഠനം മൂന്നാം അധ്യായത്തിൽ അവതരിപ്പിച്ചിരിക്കുന്നു. മെറ്റബോളിക് വിശകലനത്തിൽ ഫ്ലേവനോയ്ഡുകൾ, ഓർഗാനിക് ആസിഡുകൾ, അമിനോ ആസിഡുകൾ, പോളിയോളുകൾ, ഫാറ്റി ആസിഡുകൾ എന്നിവയുടെ ഗണ്യമായ വർദ്ധനവ് കണ്ടെത്തി. ഇവ ലോഹചീലനം, ഓക്സിലേറ്റീവ് സമ്മർദ്ദ ശമനം, റെഡോക്സ് ബാലൻസ് നിലനിർത്തൽ എന്നിവയ്ക്ക് നിർണായകമാണ്. ലോഹ സംവഹനം, ചീലനം, ആന്റിഓക്സിഡന്റ് പ്രതിരോധം, സ്ട്രെസ് സിഗ്നലിംഗ് എന്നിവയുമായി ബന്ധപ്പെട്ട ജീനുകളുടെ പ്രകടനത്തിലെ വർദ്ധനയും പ്രകാശസംശ്ലേഷണവുമായും കോശഭിത്തിയുടെ പുനർഘടനയുമായും ബന്ധപ്പെട്ട ജീനുകളുടെ പ്രകടനത്തിലെ കുറവുമാണ് ട്രാൻസ്ക്രിപ്റ്റോമിക് പ്രൊഫൈലിംഗിൽ ശ്രദ്ധിക്കപ്പെട്ടത്. ഇലകൾ, തണ്ടുകൾ, വേരുകൾ എന്നിവയിലുടനീളം തിരഞ്ഞെടുത്ത എട്ട് ജീനുകളുടെ RT-PCR സാധൂകരണം ഈ ഫലങ്ങളെ പിന്തുണച്ചു. വേരുകളിലും ഇലകളിലും HIPP39, ABCC2, GST, YSL1, PCS എന്നിവയുടെ ശക്തമായ പ്രേരണം കണ്ടെത്തി, അതേസമയം XTH, LAC6, CASP എന്നിവ ദീർഘകാല Cr സാന്നിധ്യത്തിൽ കുറഞ്ഞു. ഈ കണ്ടെത്തലുകൾ സംയുക്തമായി എ. ടെനെല്ലയിലെ ക്രോമിയം പ്രതിരോധത്തിന്റെ തന്മാത്രാ, ഫിസിയോളജിക്കൽ അടിസ്ഥാനത്തെക്കുറിച്ച് നന്നായി മനസ്സിലാക്കാനും സസ്യ-ലോഹ ഇടപെടലുകളെക്കുറിച്ചുള്ള ധാരണയും നൽകുന്നു. ക്രോമിയം ഹൈറ്റോഎക്സ്‌ട്രാക്ഷനുള്ള എ. ടെനെല്ലയുടെ കഴിവും മലിനമായ നദീതീര ആവാസ വ്യവസ്ഥകളിലെ അതിന്റെ പാരിസ്ഥിതിക ആധിപത്യവും അതിനെ ഹൈറ്റോറെമീഡിയേഷനും ആവാസവ്യവസ്ഥ പുനഃസ്ഥാപനത്തിനുമുള്ള ഒരു വാഗ്ദത്ത സസ്യമാക്കി മാറ്റുന്നു.

സൂചക പദങ്ങൾ: റിപ്പേറിയൻ, അമരന്തേസി, ഡിറ്റോക്സിഫിക്കേഷൻ, ട്രാൻസ്ക്രിപ്റ്റോമിക്സ്, മെറ്റബോളമിക്സ്, ഹൈറ്റോസോഷ്യോളജി.

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ABBREVIATIONS

4CL	-	4 coumarate CoA-ligase
A	-	Abundance
A/F	-	Abundance / frequency
ABA	-	Absciscic acid
ABC	-	ATP-binding cassette
ACO	-	ACC oxidase
ACS	-	Aminocyclopropane-1-carboxylate synthase
ADH	-	Alcohol dehydrogenase
ADT	-	arogenate dehydratase
AI	-	Acid invertase
Al	-	Aluminium
ANOVA	-	Analysis of variance
ANS	-	Anthocyanidin synthase
APX	-	Ascorbate peroxidase
ARF	-	Auxin Response Factor
As	-	Arsenic
AsA	-	Ascorbate
ATPS	-	ATP sulfurylase
AUX1	-	Auxin-resistant 1
AVT	-	Vacuolar amino acid transporters
BAC	-	Biological accumulation coefficient
BCF	-	Bioconcentration factor
BR	-	Brassinosteroid
BSA	-	Bovine serum albumin
BTC	-	Biological transfer coefficient
BZR1/2	-	Brassinosteroid-resistant 1/2
C	-	Carbon
C3H	-	Coumarate 3-hydroxylase
C4H	-	Cinnamate 4-hydroxylase
Ca	-	Calcium
CAD	-	Cinnamyl alcohol dehydrogenase
CASPs	-	Casparian strip membrane proteins
CAT	-	Catalase

CCoAOMT	-	Caffeoyl CoA O-methyl transferase
Cd	-	Cadmium
CDFs	-	Cation Diffusion Facilitators
CEQORH	-	Chloroplastic oxoene reductase
CHI	-	Chalcone isomerase
Chl a	-	Chlorophyll a
Chl b	-	Chlorophyll b
CHS	-	Chalcone synthase
Cl	-	Chlorine
Co	-	Cobalt
COI1	-	Coronatine-insensitive protein 1
CPK	-	Calcium protein kinase
Cr	-	Chromium
CS	-	Cellulose synthase
Cu	-	Copper
CYCD3	-	Cyclin D3
CYPs	-	Cytochrome P450
D	-	Density
DAMs	-	Differentially abundant metabolites
DD	-	Dominance-distribution
DEGs	-	Differentially expressed genes
DHAR	-	Dehydroascorbate reductase
DREB	-	Dehydration-responsive element binding
EDXMA	-	Energy dispersive X-ray microanalysis
EIN3	-	Ethylene-insensitive 3
EPA	-	Environmental Protection Agency
ETH	-	Ethylene
F	-	Frequency
F3H	-	Flavonone 3-hydroxylase
FC	-	Fold changes
Fe	-	Iron
FLS	-	Flavonol synthase
FT-IR	-	Fourier-transform infrared
GA3	-	Gibberellic acid
GA3ox	-	Gibberellin 3-beta-dioxygenase
GAE	-	Gallic acid equivalent

GCS	-	Gamma-glutamyl cysteine synthetase
GGT	-	Gamma-glutamyl transpeptidase
GH	-	Glutathione hydrolase
GID1	-	GA-INSENSITIVE DWARF1
GME	-	GDP-mannose 3,5-epimerase
GPI	-	Gross polluting industries
GPX	-	Guaiacol peroxidase
GR	-	Glutathione reductase
GRBMP	-	Ganga River Basin Management Plan
GSH	-	Glutathione
GSS	-	Glutathione synthetase
GSTs	-	Glutathione S-transferases
GVA	-	Gross value added
H ₂ O ₂	-	Hydrogen peroxide
HCT	-	Shikimate O-hydroxycinnamoyltransferase
Hg	-	Mercury
HIPPs	-	Heavy metal-associated isoprenylated plant proteins
HM	-	Heavy metal
HMA	-	Heavy-metal-transporting ATPase
HSFs	-	Heat shock factors
HSPs	-	Heat shock proteins
IAA	-	Indole-3-acetic acid
IAPS	-	Invasive alien plant species
IARC	-	International Agency for Research on Cancer
IPA	-	Indole-3-pyruvic acid
IPNI	-	International Plant Names Index
IR	-	Infrared
ISP	-	Ionic soluble pectin
IVI	-	Important Value Index
JA	-	Jasmonic acid
K	-	Potassium
KEGG	-	Kyoto Encyclopedia of Genes and genomes
LAC	-	Laccase
LC-MS/MS	-	Liquid chromatography and tandem mass spectrometry
LHC	-	Light-harvesting complex
LoC	-	Leaf control

LoT	-	Leaf treated
MAPK	-	Mitogen-activated protein kinase
MATE	-	Multidrug and Toxic Compound Extrusion
MDA	-	Malondialdehyde
MFS	-	Major facilitator superfamily
Mg	-	Magnesium
MKK	-	Mitogen-activated protein kinase kinase
Mn	-	Manganese
MT	-	Metallothioneins
MTP	-	Metal tolerance proteins
N	-	Nitrogen
Na	-	Sodium
NA	-	Nicotianamine
NADPME	-	NADP-dependent malic enzyme
NAS	-	Nicotianamine synthase
NBT	-	Nitroblue tetrazolium
NPT	-	Sodium Phosphate transporters
NQO1	-	(NADP)H quinone oxidoreductase 1
NRAMPs	-	Natural resistance-associated macrophage proteins
O	-	Oxygen
O ₂ ⁻	-	Superoxide radicals
OEC	-	Oxygen-evolving complex
P	-	Phosphorus
PAE	-	Pectin acetylerase
PAL	-	Phenylalanine ammonia-lyase
PAST	-	Paleontological Statistics Software Package
Pb	-	Lead
PC	-	Phosphatidylcholine
PC	-	phytochelatin
PCA	-	Principal component analysis
PCR	-	Plant Cadmium Resistance
PDCB	-	Plasmodesmata callose-binding protein
PDH	-	Proline dehydrogenase
PDR	-	Pleiotropic drug resistance
PEPC	-	Phosphoenolpyruvate carboxylase
PFAM	-	Protein family

PG	-	Polygalacturonase
PGPB	-	Plant growth-promoting bacteria
PHT	-	Phosphate transporters
PIF	-	Phytochrome-interacting factor
PLS-DA	-	Partial least squares-discriminant analysis
PME	-	Pectin methylesterase
PMEI	-	Pectin methylesterase inhibitors
POD	-	Peroxidase
POWO	-	Plants of the world online
POX	-	Plant peroxidase
PP2C	-	Phosphatase 2C
PPDK	-	Pyruvate orthophosphate dikinase
PPO	-	Polyphenol oxidase
PR	-	Pathogenesis-related protein
PSI	-	Photosystem I
PSII	-	Photosystem II
PVPP	-	Polyvinylpyrrolidone
RBOH	-	Respiratory burst oxidase
RNA-seq	-	RNA sequencing
RND	-	Resistance-nodulation-cell division
RoC	-	Roots control
ROS	-	Reactive oxygen species
RoT	-	Roots treated
Rubisco	-	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RWC	-	Relative water content
S	-	Sulphur
SA	-	Salicylic acid
SAM	-	S-adenosyl methionine synthetase
SAT	-	Serine acetyltransferase
SE	-	Standard error
SEM	-	Scanning electron microscope
sHSPs	-	Small heat shock unigenes
Si	-	Silicon
SoC	-	Stem control
SOD	-	Superoxide dismutase
SoT	-	Stem treated

SUS	-	Sucrose synthase
SWEET	-	Sugars Will Eventually be Exported transporters
TBA	-	Thiobarbaturic acid
TCA	-	Trichloroacetic acid
TF	-	Translocation factor
TFs	-	Transcription factors
Trp	-	Tryptophan
V	-	Volume
VIP	-	Variable importance in projection
VITs	-	Vacuolar iron transporters
W	-	Weight
WHO	-	World Health Organization
XET	-	Xyloglucan:xyloglucosyl transferase
XTH	-	Xyloglucan Endotransglucosylase/Hydrolase
ZIP	-	ZRT, IRT-like proteins
Zn	-	Zinc

1.1. Industrial Pollution and Heavy Metals

Increased industrialization and urbanization have accelerated the rate of environmental pollution with heavy metals, pesticides, microplastics, petroleum products, and persistent organic pollutants (Liu *et al.*, 2022). Among these, heavy metals (HMs) are a major class of inorganic contaminants that are toxic to the living system. While natural processes such as volcanic eruptions and the geological weathering of metal-containing rocks release heavy metals into the environment, anthropogenic activities including mining, combustion, improper waste disposal and agricultural practices contribute significantly to their increased levels.

Heavy metals are metallic elements with high density ($> 5.0 \text{ g/cm}^3$), high atomic weight (> 65) and toxic even at low concentrations (like ppm or ppb). HMs act as pollutants contaminating ecosystems and posing toxicity risks to living organisms, despite their natural occurrence in the environment. Many HMs are chemically and biologically stable, hence they do not degrade over time and accumulate in the environment often reaching harmful concentrations. Heavy metals can be classified as essential and non-essential. HMs that are vital for the growth, metabolism and development of living organisms are the essential ones. This includes metals such as copper (Cu), iron (Fe), manganese (Mn), cobalt (Co), zinc (Zn) and aluminium (Al), which play key roles in plant growth and development. These metals can become toxic to plants at increased concentrations. Non-essential HMs such as arsenic (As), mercury (Hg), cadmium (Cd), chromium (Cr) and lead (Pb) have no known biological function in living organisms. These metals are toxic even at low concentrations and can accumulate in living tissues, causing adverse effects.

The incremental nature, persistence, and non-degradability of HMs cause irreversible harm to ecosystems and human health. These metals are difficult to transform into non-toxic forms under natural conditions and their indiscriminate discharge into the environment results in long-lasting effects on ecosystems and pose a serious threat to human health on a global scale (Chen *et al.*, 2020). They are cytotoxic, mutagenic, carcinogenic in nature, and can cause genomic instability and

epigenetic alterations therefore even very small doses of these substances can have major health effects and can even be fatal (Balali-Mood *et al.*, 2021; Dixit *et al.*, 2015).

1.2. Heavy metals and their impact on plants

Heavy metals disrupt the normal biochemical and physiological functions of plants, impairing their growth, development, and productivity (Fig. 1.1). At higher concentrations, HMs such as Pb, Cd, Cr, and Hg interfere with the metabolic pathways involved in respiration, photosynthesis, nutrient uptake, water regulation, and enzymatic activities (Angon *et al.*, 2024). This interference causes stunted plant growth, chlorosis, wilting, biomass reduction and even plant death. The reactive oxygen species (ROS) generated by HMs cause oxidative stress in plants that can ultimately impact plant growth and crop productivity through cell membrane damage, protein degradation and DNA alterations (Singh and Kalamdhad, 2011).

Heavy metals have indirect effects on plants such as disrupting their beneficial interactions with soil microorganisms. For example, HMs negatively influence the ability of mycorrhizal fungi that form symbiotic associations with plant roots to aid in nutrient and water uptake (Gladkov *et al.*, 2023). This disruption reduces nutrient uptake and impairs the ability of plant to withstand environmental stresses. Water stress is a significant environmental stress imparted by that affects especially the plants in arid and semi-arid regions. Water stress adversely affects physiological processes such as photosynthesis, impairing plant growth and survival (Chaves *et al.*, 2002).

Heavy metals also contribute to nutritional imbalances in plants. Metals like Pb and Cd mimic essential nutrients and compete for uptake by plant roots. This competition often results in nutrient deficiencies since toxic metals are absorbed more efficiently than essential elements. Hence, plants suffer from disrupted reproductive processes, reduced growth and lower crop quality and yield (Angon *et al.*, 2024). HMs can also affect the availability of essential nutrients by altering soil chemistry. They restrict the nutrient availability to plants by binding to soil

particles or by forming insoluble complexes. This enhances nutrient deficiencies that ultimately reduces the plant development and productivity. Thus, plants grown in metal-contaminated environments show decreased crop yields and raise concern about food security and environmental sustainability.

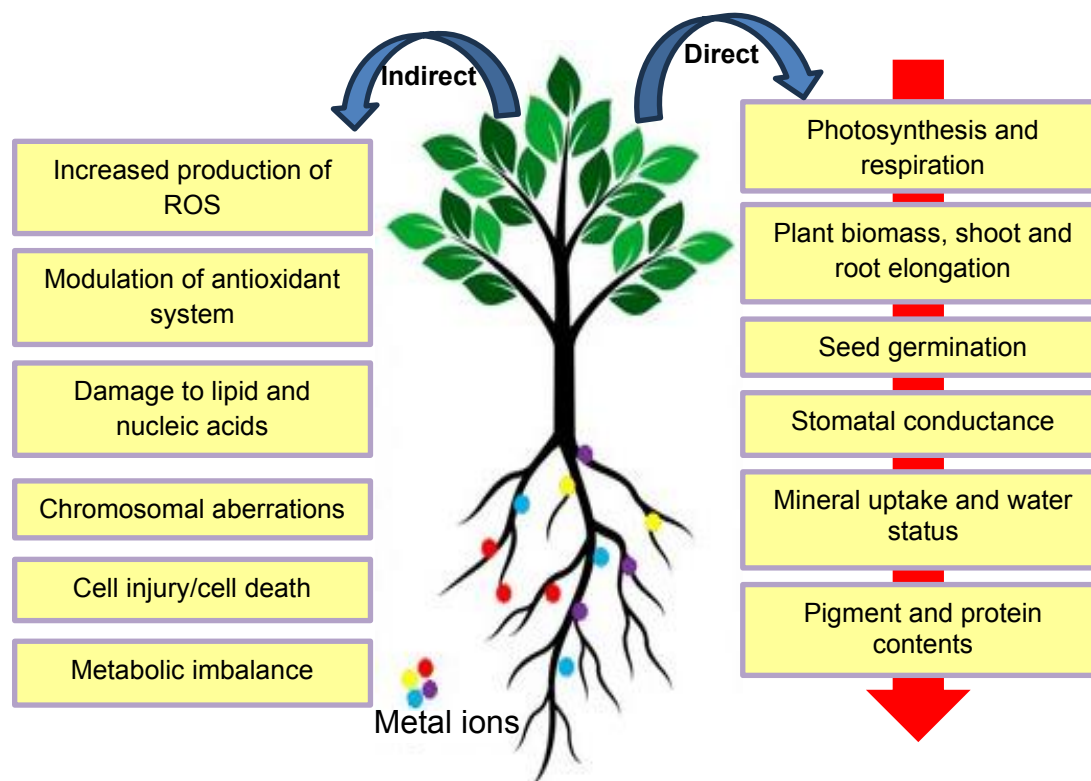


Fig 1.1. Effect of heavy metals on plant growth and physiology.

High concentrations of HMs in the soil are absorbed by plants, translocated to their shoots, and subsequently accumulate and biomagnify through higher trophic levels in the food chain, posing a serious risk to animal and human health (immune system and kidney dysfunction, damage to nervous and cardiovascular system, carcinogenic effects). Hence, developing effective methods for removing heavy metals from the environment is essential.

1.3. Techniques for removal of heavy metals

Natural processes like leaching, plant absorption, erosion, and deflation can cause heavy metal depletion in the environment, but the efficiency is low (Chen *et al.*,

2020). Many physio-chemical processes, such as reverse osmosis, chemical precipitation and oxidation/reduction, ion exchange, adsorption, filtration, solvent extraction, and electrochemical treatment, were developed to eliminate the HMs from the contaminated environment (Levchuk *et al.*, 2018; Burakov *et al.*, 2018; Liu *et al.*, 2018; Huang *et al.*, 2017; Al-Alawy and Al-Ameri, 2015). But these techniques are less sustainable and require high cost and maintenance. Alternately, highly efficient biosorption and/or hyper-bioaccumulation are promising biological remediation strategies for HM removal that could restore and maintain the natural balance of the environment (Liu *et al.*, 2020; Liu *et al.*, 2018). Plants and microorganisms are frequently used as acceptors in biological methods (Dixit *et al.*, 2015).

1.4. Phytoremediation: A Sustainable Solution

Phytoremediation has emerged as a promising alternative to conventional remediation techniques (Salt *et al.*, 1998). Plants and their associated soil microbes are used in this method to remove or reduce elemental pollutants from soil, air, and water by establishing a rhizosphere ecosystem that absorbs ionic compounds (HMs), thereby cleaning up contaminated environments and stabilizing soil fertility. This green technology utilizes the natural ability of certain plants (hyperaccumulators) to accumulate, immobilize or detoxify pollutants in soil and water. It offers advantages such as sustainability, environment-friendly, applicability to large areas, and low operational costs (Liu *et al.*, 2020). Ideal phytoremediation candidates should exhibit strong growth, high biomass, extensive root systems, inedibility and hyperaccumulation of contaminants with stress tolerance. Hyperaccumulators, such as *Brassica juncea*, *Ricinus communis*, *Jatropha curcas*, *Solanum nigrum* and *Arabidopsis halleri* have shown great potential in removing toxic metals (Skuza *et al.*, 2022). However, the slow growth rate of plants and site-specific limitations pose challenges that need further research.

Plants employ various mechanisms to remediate heavy metal contamination, including according to the type of soil and pollutant, species of the plant etc., as follows:

- a) **Phytoextraction:** Phytoextraction or phytoaccumulation is a process where plants absorb dissolved HMs through their roots. To facilitate absorption, the metals must dissolve in substances that can be taken up by the plant. Plants defend themselves and enhance metal mobility by chelating the metals, binding them to organic molecules either before or after absorption. Once chelated, the metals are transported to specific plant tissues for storage, which can be harvested and removed. *Commelina communis* for Cu, *Xanthium strumarium* for Cd, Pb, Ni, Zn, *Brassica juncea* for Pb, *Pteris vittata* for As are some hyperaccumulator plants that can accumulate high concentrations of metals (Salido *et al.*, 2003; Yang *et al.*, 2019; Wang *et al.*, 2004).
- b) **Phytostabilization:** It involves the immobilization of metals within the root or the rhizosphere and decreasing the solubility or bioavailability of contaminants in soil, preventing their spread. Plant roots aid immobilization by depositing, absorbing, or precipitating contaminants within the root zone. The conjugation of HMs with derivatives of sugars, proteins, and amino acids can convert them into non-toxic compounds. *Festuca rubra* for Cu, Pb, Mn, and Zn (Touceda-González *et al.*, 2017) and *Boehmeria nivea* for Cd, Pb (Lan *et al.*, 2020) are reported as potential plant species for phytostabilization.
- c) **Phytovolatilization:** Phytovolatilization involves the uptake of contaminants by plants from the soil, converting them into volatile forms, and releasing them into the atmosphere. For example, mercury can be converted to a less toxic gaseous form (Meagher and Heaton, 2005). This method is most effective for organic pollutants that become less toxic upon volatilization. Contaminants can volatilize directly from plant stems and leaves or because of root-soil interactions. *Polypogonmon speliensis* and *Scirpus robustus* are capable of volatilizing As and Se respectively through volatilization mechanism and releasing it to the atmosphere (Ruppert *et al.*, 2013; Arthur *et al.*, 2005).
- d) **Rhizofiltration:** It is a phytoremediation technique that uses plant roots to absorb and filter contaminants from water. It involves adsorption, absorption, and concentration, precipitation of pollutants such as HMs and nutrients onto

or within the roots of aquatic plants. Aquatic plants are commonly employed due to their ability to thrive in water-saturated conditions and effectively remediate nutrient-rich or metal-contaminated environments. For HM contamination, roots can facilitate the precipitation of metals into insoluble forms, trapping them within root tissues. Some plants that are known to utilize rhizofiltration during phytoremediation are *Eichhornia crassipes* for Fe, Cr, Cu, Cd, Zn, Ni, As (Rai, 2019), *Azolla caroliniana*, *Callitriche lusitanica*, and *Ranunculus trichophyllus* for As (Favas *et al.*, 2012), and *Typha angustifolia* for Cu, Pb, Ni, Fe, Mn, and Zn (Chandra and Yadav, 2010).

- e) Phytodegradation and rhizodegradation: Plants metabolize and break down pollutants such as HMs into less harmful compounds within their tissues through a process called phytodegradation. Contaminants are absorbed through passive uptake and degraded directly by the plant. Rhizodegradation occurs in the rhizosphere, where plant roots stimulate microbial activity that breaks down contaminants in the surrounding soil. Both processes are effective for HM remediation, since phytodegradation acts within plant tissues and rhizodegradation relies on root-soil interactions. *Sesbania cannabina* is known for phytodegradation of Pb (Maqbool *et al.*, 2012).

Each of these mechanisms involve complex interactions between plants, soil and microorganisms which makes phytoremediation a multidisciplinary field of study.

1.5. Invasive plants for phytoremediation

Invasive alien plant species (IAPS) are one such category of plants that are most suitable for phytoremediation of polluted soils in industrial areas, especially riparian zones. These plants exhibit unique attributes such as rapid growth, extensive root systems and the ability to accumulate high concentrations of HMs in their tissues without suffering from phytotoxic effects (Liu *et al.*, 2013; Oliveira, 2012). They thrive in barren, contaminated environments and often outcompete native plants due to their adaptability, competitiveness and fecundity (Prabakaran *et al.*, 2019). The robust root systems and symbiotic microbial associations further enhance their capacity to absorb and stabilize pollutants in the soil (Wang *et al.*, 2020; El-Bakatoushi and Elframawy, 2016). These traits enable IAPS to flourish in

areas impacted by anthropogenic activities which makes them valuable candidates for remediating metal-polluted soils especially in riparian zones (Wei *et al.*, 2018; Yang *et al.*, 2020). Invasive plants, such as *Impatiens glandulifera*, *Reynoutria japonica*, *Solidago canadensis*, *Amaranthus* spp. are reported for their phytoremediation potential (Miletić *et al.*, 2024; Zhou *et al.*, 2024). Despite their potential, research on the mechanisms and efficiency of invasive plants in phytoremediation remains limited and demands further exploration.

1.6. Conclusion and research scope

Industrial pollution and heavy metal contamination pose significant environmental challenges that demand innovative and sustainable remediation strategies. Phytoremediation using invasive plants offers an effective and sustainable solution because of their adaptability, high biomass production and capacity to accumulate contaminants. However, understanding the mechanisms behind their tolerance and detoxification processes is crucial for optimizing their application in remediation.

This study aims to contribute to this field by identifying a potential plant species for phytoremediation and exploring its morphological, anatomical, physiological, and molecular responses to heavy metal stress. The findings will provide valuable insights into the mechanisms of heavy metal detoxification, potentially enhancing the effectiveness of phytoremediation. Each of these objectives is discussed in detail in the subsequent chapters, forming a comprehensive investigation into the role of plants in mitigating heavy metal pollution.

1.7. Objectives of the study

- i) Analysis of the riparian flora in Kanjikode, Palakkad district and to screen native plant species growing wild in these sites for heavy metal uptake.
- ii) Characterization of heavy metals uptake by selected plant with reference to morphological, anatomical and biochemical changes.
- iii) Identification of metabolites and pathways involved in metal detoxification using LC-MS and de novo transcriptome analysis.

2.1. Introduction

The promising economic growth offered by industrialization significantly accelerates environmental pollution in coupling with urbanization and population growth. India, which ranks fifth in the global economy is fueled by the industrial sector which contributes about 30 percent of the total gross value added (GVA) in the country. The rapid increase in gross polluting industries (GPIs) in India by 136 percent over the past decade deteriorates the water, air and land quality (SoE, 2023). GPIs include metal processing, textile units, tanneries, chemical units, food, dairy and beverage industries that discharge more than 100,000 liters of effluent water with hazardous chemicals into the rivers, contaminating the soil, surface and groundwater (Hussain *et al.*, 2014; Nazir *et al.*, 2011). Rivers, in addition to being used for domestic, agricultural, power and industrial purposes, are now grossly polluted with industrial and sewage wastes, often exceeding their natural assimilative capacities (Baskaran and Britto, 2010). Industrial effluents are a rich source of heavy metals [cadmium (Cd), chromium (Cr), lead (Pb), aluminum (Al), copper (Cu), etc.] that degrade surrounding soil and water ecosystems, putting riparian vegetation under constant metal contamination stress.

The United States Environmental Protection Agency (EPA 2005) defined riparian areas as “the vegetated ecosystems along water body through which energy, materials and water pass”, while Kocher and Harris (2007) defined riparian areas as the areas adjacent to rivers. These ecosystems play a key role in purifying pollutants, primarily through plants that absorb and decompose harmful substances. Thus, such discharges exert a strong influence on the environmental, physical and chemical processes that shape riparian vegetation which provides an opportunity for environmental monitoring using plants. Riparian vegetation reflects high diversity in species and influences various ecological functions including sedimentation rate, stream flow rates, recharged water tables, stream bank stabilization, diverse animal assemblages and ecological corridors (Richardson *et al.*, 2007; Sakio and Tamura, 2008; Naiman *et al.*, 2010; Fremier *et al.*, 2015). However, human mediated disturbances at local to global scales affect

riparian ecosystem (Richardson *et al.*, 2007). Anthropogenic activities including mining, farming, grazing, logging, constructions, urban and industrial developments are the major reasons for the degradation of riparian areas and thereby reducing biodiversity (Vallari *et al.*, 2009). This facilitates the colonization of allochthonous plants, which outcompete existing species. The shifts in the abundance of dominant species consequently result in significant functional changes (Hooper *et al.*, 2005). These functional changes are higher in case the abundance of the dominant species changes; but when a highly dominant species was present, the riparian diversity was found to be very low (Hejda and Pysek, 2006). Based on this rationale, it has been hypothesized that effluent discharges from industries can modify the floristic diversity of riparian vegetation.

A gradual shift in the structure and composition of vegetation can be seen at sites that continuously receive polluted water (Ali *et al.*, 2004). The pollutants can influence the species richness and cover of the plant positively or negatively. A flora catalogues all plant species within a geographical area and reflects the diversity, resource patterns, biota and environmental influences of the specific region (Berrens, 2019). Comprehensive field surveys enable the identification of dominant native plants that adapt well to contaminated areas (Boruah *et al.*, 2020). Hence, floral inventories are important in ecological restoration efforts. Phytosociological studies provide an account on the floristic vegetation characteristics of an area encompassing the species richness, distribution of species, and diversity. These studies employ diversity indices like the Shannon-Weiner index to compare species abundance and diversity in disturbed areas (Feranchuk *et al.*, 2018). Herbs play a key role in the species diversity of a vegetation and respond quickly to environmental changes, making them good indicators of pollution levels (Sagar *et al.*, 2012). The tolerance or sensitivity of individual species determines how their distribution and resource use are influenced by pollutants in the habitat (Shannigrahi *et al.*, 2004). Native or wild plant species that thrive in polluted environments can also be instrumental in remediating contaminated soils through phytoremediation.

Despite evidence of heavy metal contamination in rivers like Periyar, Pamba, Karamana, and Ithikkara (Mathew and Radhika, 2022; Prasood *et al.*, 2024; Pournami and Sobha, 2022), little research has focused on the impact of industrial pollution on riparian vegetation in Kerala. Kanjikode in the Palakkad district is the second largest industrial area in Kerala. The Walayar River traversing through Kanjikode and its adjoining areas experience intense anthropogenic disturbances and pollution from continuous industrial effluent discharge. However, no phytosociological studies have been conducted on the riparian vegetation of the Walayar River which leaves a gap in understanding how these pollutants impact plant diversity and ecosystem health.

Chronic exposure to heavy metals even at low doses can disrupt biological processes leading to altered community structures and ecosystem dynamics (Mussali-Galante *et al.*, 2014). Plants in these polluted environments can evolve metal tolerance that makes them useful for phytoremediation. The metal tolerant plants can be identified by assessing the floristic diversity and phytosociology of herbaceous species in contaminated sites like Walayar. These species may serve as pollution indicators and potential tools for soil remediation from heavy metals. Metal bioconcentration factor (BCF), biological transfer coefficient (BTC) and biological accumulation coefficient (BAC) is used for assessing the heavy metal remediation efficiency of plants. Therefore, the study aimed to investigate the floristic diversity and phytosociology of the riparian vegetation along the Walayar River, to assess the effects of industrial contaminants on this ecosystem and to explore the phytoremediation potential of dominant plant species.

2.2. Review of literature

2.2.1. Riparian vegetation and its ecological role

Riparian vegetation refers to plant habitats and communities that grow along margins, banks and floodplains. They are unique and highly dynamic ecosystems rich in biodiversity. Naiman *et al.* (2005) defined riparian zones as transitional areas between aquatic and terrestrial ecosystems where the vegetation plays an

important role in maintaining the riparian functions. Riparian vegetation stabilizes riverbanks, provides nutrients, mitigates erosion, regulates temperature and water quality (Stackhouse *et al.*, 2023). These zones act as buffer systems by absorbing excess nutrients and pollutants, which is important especially in areas affected by anthropogenic activities such as industrial development (Sweeney *et al.*, 2004). The rapid expansion of industries, waste production, agriculture and urban development has severely degraded riparian areas by exposing plants to pollutants such as heavy metals, hydrocarbons, microplastics, particulate matter and nutrients like nitrates and phosphates and (Napoletano *et al.*, 2023). These plants act as bioindicators that reflect the health of the environment and its level of pollution. Some plants can survive and adjust to industrial pollution which makes them ideal for phytoremediation, a process where plants absorb, detoxify and restore contaminated soils and environments (Pulford and Watson, 2003).

2.2.2. Methods and Applications of Phytosociological Studies

Phytosociology or phytocoenology is a branch of vegetation science that studies plant communities of specific vegetation zones to determine their structure, composition, evolution and species relationships (Braun-Blanquet, 1964). It helps show the dynamics of plant cover such as species richness, diversity, dominance, and adaptability especially in stressed environments (Konatowska and Rutkowski, 2019). Phytosociological studies in riparian zones are essential for understanding how vegetation patterns change under environmental stresses such industrial pollution. To study these patterns, researchers often use quadrat sampling to measure species abundance and dominance. They also use diversity indices such as Shannon-Weiner and Simpson indices to assess species diversity and the Importance Value Index (IVI) to determine the relative dominance and ecological importance of plant species (Curtis and McIntosh, 1950). These studies are used worldwide to understand the resilience of vegetation in degraded environments and help guide conservation and restoration efforts for disturbed ecosystems.

2.2.3. Riparian vegetation studies

The species richness, diversity and phytosociology of riparian vegetation have been widely studied in rivers across Asia, Europe, America, Africa and Australia. Extensive research on riparian diversity has been conducted in Walga river (Meragiaw *et al.*, 2018), Taquari river (Lucheta *et al.*, 2018), Wami River (Milgo, 2017), Danube River (Molder and Schneider, 2011), Miranda River (Wittmann *et al.*, 2008), Macal River (Urban *et al.*, 2006), Queets River (Balian and Naiman, 2005), Willamette River (Fierke and Kauffman, 2005), La Plata River (Heartsill-Scalley and Aide, 2003), Missouri River (Johnson, 2002), Biebrza River (Wassen *et al.*, 2002), Boyer River (Boutin *et al.*, 2002), Caura River (Rosales *et al.*, 2001), and Andarax River (Salinas *et al.*, 2000). In Indian context, research on diversity of riparian vegetation has been conducted by Nagarajan and Bhaskar (2023) in Moyar River, Mohanan and Thomas (2022) in lower stretches of Chaliyar River, Paul *et al.* (2021) in Pamba River, Kujur *et al.* (2022) in Maini River and Machhli River, Ali *et al.* (2022) in Chenab River, Sakthivel *et al.* (2019) in Amirthi River, Cherullipadi and Paul (2016) in Bharathappuzha River, Vincy *et al.* (2015) in Meenachil River, Sunil *et al.* (2010) in Cauvery River, Kumara and Srikantaswamy (2011) in Tungabhadra River, Sreedharan (2005) in Valapattanam River, Chauhan and Gopal (2005) in Yamuna River, and Bachan (2003) in Chalakkudy River. None of them discussed the impact of industrial activities on the distribution and diversity of riparian vegetation.

2.2.4. Riparian Vegetation and Industrial Pollution

Pollutants from industrial areas significantly impact riparian vegetation by altering soil chemistry, water quality, and microclimatic conditions. The contamination of soil with HMs such as Cd, Hg, Zn, Cr and Pb from industrial activities often leads to soil acidification (Dehkordi *et al.*, 2024). The discharge of toxic chemicals and untreated pollutants into water sources make the water unsafe for marine life and human use (Earth.org, 2022). Changes in canopy temperature, soil moisture, wind speed, rate of water loss and light interception further disrupts the microclimatic conditions in industrial areas (Kingra and Kaur, 2017). Previous studies have shown the impact of industrial pollution on plant species. Heavy metal

contamination in soil can slow plant growth, but it can also help in identifying plants that are tolerant to such conditions (Ali *et al.*, 2013). Industrial pollution often reduces the population of sensitive species, while more tolerant species (such as *Ricinus communis*, *Celosia argentea* and grasses like *Apluda mutica*) proliferate (Singare and More, 2020). These tolerant plants have advanced adaptations that help them survive. These include physical barriers such as cuticle, lenticels, strengthened cell walls and physiological mechanisms like synthesis of osmolytes and osmoprotectants, vacuolar sequestration of metals, enhancing antioxidant systems, activating stress responsive genes, reducing metal uptake and translocation (Ahmad and Prasad, 2011). Research from industrial regions around the world emphasizes the importance of native and resilient species in reclaiming industrial wastelands. These plants help to restore the ecological balance and support the development of self-sustaining ecosystems (Gairola *et al.*, 2023).

2.2.5. Relevant Studies in the Indian Context

Being a rapidly developing nation with a growing population, India faces major challenges due to industrial pollution. Several studies have investigated the impact of industrial activities on riparian and other types of vegetation. The Ganga River Basin Management Plan (GRBMP, 2012) developed by IITs in India has reported how riparian vegetation in the Ganga River basin adapts to different pollution levels. Rai (2009) reviewed the use of macrophytes such as *Typha* spp., *Potamogeton* spp., *Eichhornia crassipes* and *Vetiveria zizanioides* for phytoremediation and emphasized their tolerance to industrial effluents and heavy metal contamination.

Khatun *et al.* (2016) documented the dominance and resilience of *Colocasia esculenta* and *Scirpus articulatus* in polluted riparian zones. Sojitra *et al.* (2024) explored the phytoremediation potential of plants from the families Cyperaceae, Poaceae and Typhaceae along contaminated riverbanks. Ghosh and Manchanda (2019) reported the ability of *Tagetes patula*, *Bassica scoparia*, and *Portulaca grandiflora* to remove heavy metals from the waters of Yamuna River. Paul *et al.* (2014) documented the abundance of weeds such as *Calotropis procera*, *Parthenium hysterophorus*, *Chenopodium murale*, *Croton bonplandianum*, *Rumex dentatus*, *Amaranthus spinosus*,

Datura stramonium and *Withania somnifera* near the polluted banks of Yamuna River. These species were found to be able to phytoextract and phytostabilize heavy metals such as Zn, Cd, Cu, Cr, As, Pb and Mn. In Kerala, rapid urbanization and industrial growth have led to the release of industrial and sewage wastes into the rivers. These effluents contain a considerable amount of heavy metals like Cd, Cr, Pb, Mn, Cu, Co, Ni, and Zn. Although heavy metal pollution in rivers such as the Periyar, Chaliyar, Pamba, Chalakudy, Manimala, Meenachil, Achankovil, Neyyar, and Karamana has been well documented (Mathew and Radhika, 2022), studies on the effect of this pollution on riparian vegetation remain limited.

The Walayar River, a tributary of Bharathapuzha, flows through Kanjikode in the Palakkad district which is the second largest industrial zone of Kerala. This makes it an important site for studying the impact of industrial pollution on riparian ecosystems.

2.2.6. Scope for Research

Although research on riparian vegetation is extensive, studies on the industrial riparian zones in Southern India including Kerala are limited. The important research gaps include the identification of pollution tolerant native species, understanding their physiological and biochemical adaptations and long-term monitoring of riparian plant communities. Existing literature shows that riparian plants act as bioindicators and ecological buffers in industrial areas. Phytosociological studies also provide details on species composition, dominance and ecological resilience. The present study conducted along the riverbanks of Walayar River in Kanjikode, helps fill these gaps by identifying the dominant plant species that can survive in polluted environments. The findings contribute to conservation and ecological restoration efforts in industrial areas.

2.3. Materials and methods

2.3.1. Study area

The research was carried out at ten randomly selected stations along the banks of the Walayar River in the industrial belt of Kanjikode, located in the Palakkad district of Kerala, India. It is located within latitude 10°46'59.99" N and longitude 76°43'59.99" E, with a length of 17.7 km. Kanjikode is the second largest industrial

area in Kerala which harbours more than 200 large-scale and small-scale industries and manufacturing units, mainly dealing with iron smelting, steel rerolling, textiles, dye and paint manufacturing. Walayar River, one among the tributaries of the Kerala's second largest river Bharathapuzha flow through Kanjikode and receives high load of pollutants from the factories. The study was conducted at the following randomly sampled ten stations (Fig. 2.1).

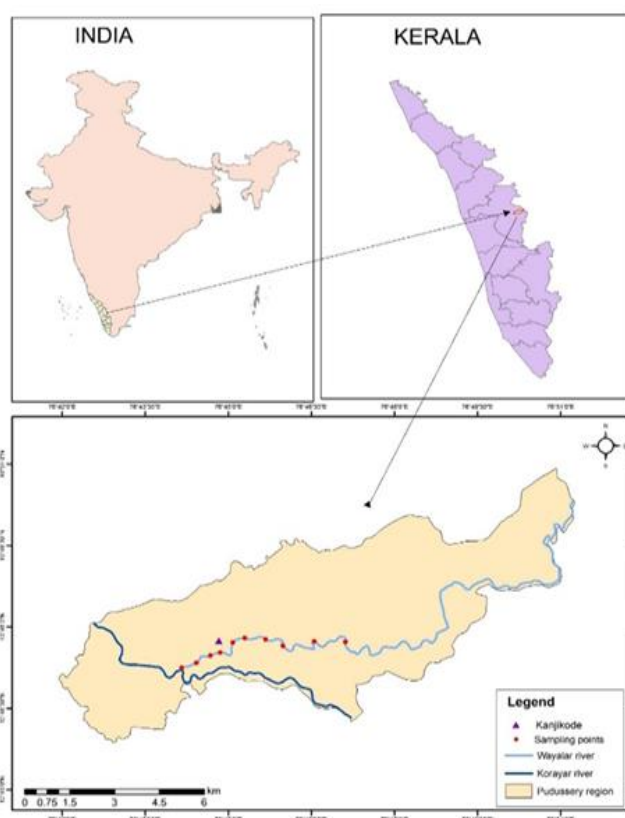


Fig. 2.1. Map of the Walayar River and the sampling points (stations 1-10) selected for phytosociological study and heavy metal analysis.

2.3.2. Vegetation sampling, species identification and data collection

The analysis of vegetation pattern and plant species distribution were undertaken to identify the dominant plant species through quadrat method of analysis. Vegetation was sampled at the ten stations using 100 randomly placed nested quadrats measuring 1m x 1m for herbaceous vegetation. The identification of specimens was facilitated by taxonomists and referenced against existing literature (Vajravelu, 1990; Sasidharan, 2004), and names were validated using the Plants of the world online (POWO, 2024) and International Plant Names Index (IPNI).

The Important Value Index (IVI) for each species was computed based on the relative values of frequency, density, and abundance, following the methodology outlined by Curtis and McIntosh (1950) and Curtis (1959). The Whitford Index was employed to reveal the distribution pattern of species through the A/F ratio (Whitford, 1948). This ratio categorized distribution as regular (<0.025), random ($0.025-0.05$), and contagious (>0.05), following the classification by Cottam and Curtis (1956). Diversity indices were calculated using the Paleontological Statistics Software Package (PAST) v.4.03.

Shannon-Wiener diversity index, H' (Shannon and Wiener, 1963)

$$H' = -\sum_{i=1}^S p_i \ln p_i$$

Simpson index of dominance, C_D (Simpson, 1949)

$$C_D = \sum_{i=1}^S (p_i)^2$$

Species richness index, SR (Margalef, 1958)

$$SR = S - 1/\ln N$$

Evenness index, e (Pielou, 1966)

$$e = H'/\log S$$

Berger-Parker Index, d (Berger and Parker, 1970)

$$d = N_{\max}/N$$

Menhinick Index, D_{mn} (Whittaker, 1977)

$$D_{mn} = S/\sqrt{N}$$

Where, p_i represents the proportional abundance of the i^{th} species (n_i/N), N is the total number of individuals of all the species, $N_{\max}$ is the number of individuals in the most important species, H' is Shannon-Wiener index and S is the total number of species.

Homogeneity/heterogeneity of the vegetational stand of the study sites is determined by recording the presence or absence of the plant species. The species types are divided into five frequency classes depending on Raunkiaer's (1934) frequency classification as below.

Class A = 1 to 20%, Class B = 21 to 40%, Class C = 41 to 60%, Class D = 61 to 80%, Class E = 81 to 100%

A histogram was drawn with percentage of the total number of species on Y- axis and frequency classes A to E on X- axis. It is compared with the law of frequency $A>B>C\geq$ or $\leq D<E$ to know whether the plant community is homogenous or heterogeneous.

2.3.3. Data analysis

Correlation analysis (similarity and dissimilarity) between each pair of sites within the study area was conducted utilizing PAST v.4.03. The analysis employed the Bray-Curtis distance measure, with clustering performed via the unweighted pair group method using arithmetic averages (UPGMA) algorithm. Pearson's correlation coefficient (r) was used to assess the relationship of the measured vegetation assemblages, using SPSS program.

2.3.4. Analysis of heavy metal content in collected soil and plant samples

Samples were randomly collected from sites where quadrats were laid, and these were mixed to form a composite whole sample. Plant samples were washed with distilled water to remove the adhered soil particles and contaminants. The shoots and roots of the plants were separated, oven-dried at 60°C until a constant weight was achieved, powdered and digested using a solution of HNO_3 : HCl (4:1 v/v) for analysis of Cr, Mn, Zn, Cu, Ni, Pb, Cd, and Co in an ICP-AES (Perkin Elmer-Aveo 200, USA) (Hansen *et al.*, 2013). Composite soil samples were air-dried, ground, homogenized using a 2mm mesh, and analysed for its heavy metal content. The phytoremediation indices such as biological accumulation coefficient (BAC), translocation factor (TF), and bioconcentration factor (BCF) were calculated using the formulas proposed by Yanqun *et al.* (2005) and Yoon *et al.* (2006).

$$\text{TF} = \frac{[\text{metal}]_{\text{shoot}}}{[\text{metal}]_{\text{root}}}$$

$$\text{BAC} = \frac{[\text{metal}]_{\text{shoot}}}{[\text{metal}]_{\text{soil}}}$$

$$\text{BCF} = \frac{[\text{metal}]_{\text{root}}}{[\text{metal}]_{\text{soil}}}$$

2.3.5. Statistical analysis

A paired t-test was employed to conduct a plant community analysis for the study sites, while one-way ANOVA test ($P < 0.01$), variance and covariance was used to compare the distribution patterns and diversity indices among the study sites.

2.4. Results

2.4.1. Floristic composition

The riparian vegetation along the Walayar Riverbank in the industrial belt of Kanjikode was more diverse and was mostly angiosperm type. A total of 97 angiospermic plant species distributed over 32 families and 83 genera was observed in the floristic composition (Fig. 2.2). The complete list of plants with their family and IVI from each station is recorded (Table 2.1). Of these, 79 were forbs and 18 species belonged to grasses and sedges. There were 27 families representing dicots, while monocots were represented by 5 families. Additionally, 13 families were poorly represented, each having only 1 species.

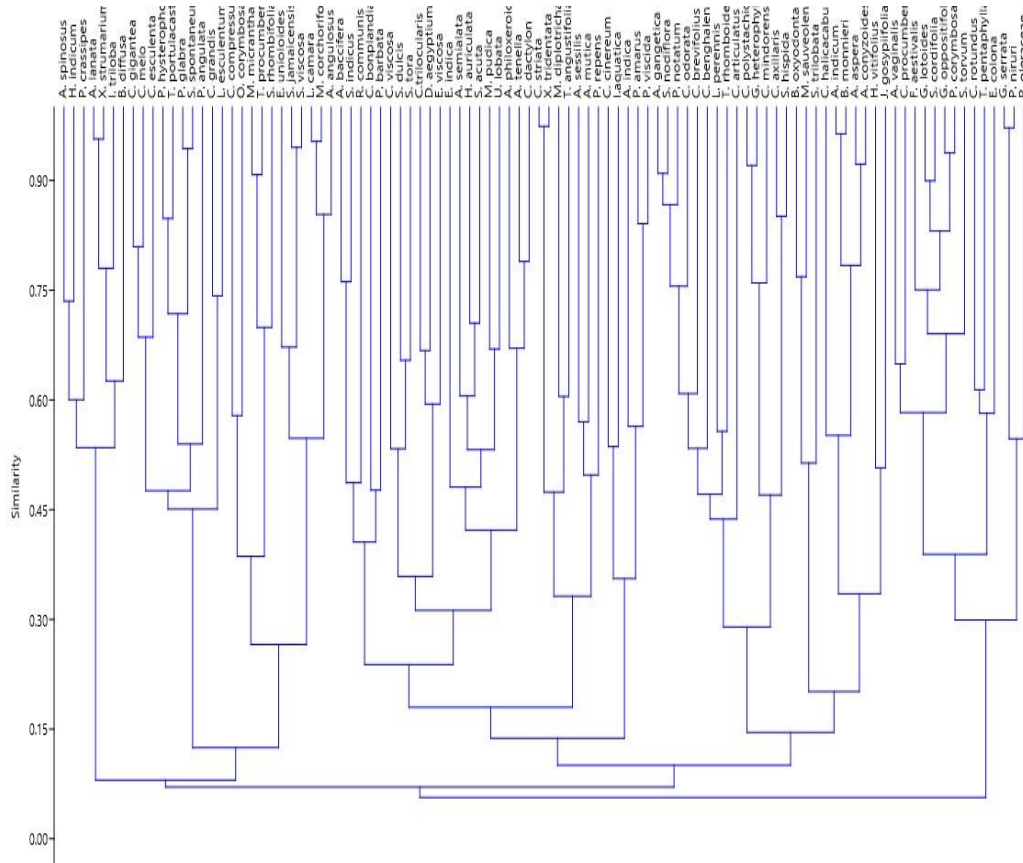


Fig. 2.2. Hierarchical clustering dendrogram representing the floristic similarity among species in the study area. Closely related species or plant groups cluster together reflecting shared ecological or morphological traits.

Table 2.1. Phytosociological analysis in different sampling stations of Walayar River.

SL. NO	NAME OF THE PLANT	FAMILY	CLASS	F	A	D	IVI	A/F
STATION 1								
1	<i>Alysicarpus vaginalis</i> (L.) DC.	Fabaceae	E	100	12.50	12.50	9.43	8.41
2	<i>Coldenia procumbens</i> L.	Boraginaceae	E	100	19.00	19.00	9.433	12.794
3	<i>Croton bonplandianus</i> Baill.	Euphorbiaceae	E	100	34.10	34.10	9.433	22.963
4	<i>Cyperus rotundus</i> L.	Cyperaceae	C	60	6.67	4.00	5.660	2.693
5	<i>Echinocloa colona</i> (L.) Link	Poaceae	D	80	10.00	8.00	7.547	5.387
6	<i>Eleusine indica</i> (L.) Gaertn.	Poaceae	C	60	21.37	12.80	5.660	8.620
7	<i>Fimbristylis aestivalis</i> (Retz.) Vahl.	Cyperaceae	B	40	17.00	6.80	3.774	4.579
8	<i>Glinus lotoides</i> L.	Molluginaceae	D	80	13.00	10.40	7.547	7.003
9	<i>Glinus oppositifolius</i> (L.) Aug.DC.	Molluginaceae	B	40	11.75	4.70	3.774	3.165
10	<i>Gomphrena serrata</i> L.	Amaranthaceae	A	20	6.00	1.20	1.887	0.808
11	<i>Panicum repens</i> L.	Poaceae	E	90	14.00	12.60	8.491	8.484
12	<i>Phyllanthus amarus</i> Schumach. & Thonn.	Phyllanthaceae	A	10	1.00	0.10	0.943	0.067
13	<i>Phyllanthus niruri</i> L.	Phyllanthaceae	A	20	5.50	1.10	1.887	0.741
14	<i>Polycarpaea corymbosa</i> (L.) Lam	Caryophyllaceae	C	60	9.67	5.80	5.660	3.906
15	<i>Portulaca oleraceae</i> L.	Portulacaceae	A	10	2.00	0.20	0.943	0.134
16	<i>Pseudarthria viscida</i> (L.) Wight & Arn.	Fabaceae	A	10	3.00	0.30	0.943	0.202
17	<i>Sida cordifolia</i> L.	Malvaceae	D	80	9.25	7.40	7.547	4.983
18	<i>Solanum torvum</i> Sw.	Solanaceae	D	70	4.71	3.30	6.604	2.222
19	<i>Trigastrotheca pentaphylla</i> (L.) Thulin	Molluginaceae	B	30	14.00	4.20	2.830	2.828
STATION 2								
1	<i>Alloteropsis semialata</i> (R.Br.) Hitchc.	Poaceae	E	100	15.30	15.30	8.330	10.160
2	<i>Alternanthera sessilis</i> (L.) R.Br. ex.DC.	Amaranthaceae	D	80	13.13	10.50	6.670	6.970

3	<i>Alternanthera tenella</i> Colla	Amaranthaceae	D	80	13.25	10.60	6.670	7.040
4	<i>Cleome viscosa</i> L.	Cleomaceae	B	40	3.50	1.40	3.330	0.930
5	<i>Corchorus trilocularis</i> L.	Malvaceae	C	60	5.83	3.50	5.000	2.320
6	<i>Crotalaria striata</i> DC	Fabaceae	C	60	3.17	1.90	5.000	1.260
7	<i>Croton bonplandianus</i> Baill.	Euphorbiaceae	B	30	3.33	1.00	2.500	0.660
8	<i>Cynodon dactylon</i> (L.) Pers.	Poaceae	E	90	19.78	17.80	7.500	11.82
9	<i>Eleusine indica</i> (L.) Gaertn.	Poaceae	B	40	14.00	5.60	3.330	3.720
10	<i>Hibiscus vitifolius</i> L.	Malvaceae	A	20	3.00	0.60	1.670	0.398
11	<i>Hygrophila auriculata</i> (K.Schum) Heine	Acanthaceae	A	20	19.50	3.90	1.670	2.590
12	<i>Ipomoea aquatica</i> Forssk.	Convolvulaceae	A	20	3.00	0.60	1.670	0.398
13	<i>Jatropha gossypifolia</i> L.	Euphorbiaceae	A	10	2.00	0.20	0.830	0.132
14	<i>Mimosa diplotricha</i> C. Wright	Fabaceae	C	50	15.00	7.50	4.160	4.980
15	<i>Mimosa pudica</i> L.	Fabaceae	B	40	18.50	7.40	3.330	4.910
16	<i>Panicum repens</i> L.	Poaceae	D	70	19.85	13.90	5.830	9.230
17	<i>Phyllanthus amarus</i> Schumach. & Thonn.	Phyllanthaceae	B	40	3.25	1.30	3.330	0.863
18	<i>Pseudarthria viscida</i> (L.) Wight & Arn.	Fabaceae	B	30	4.67	1.40	2.500	0.930
19	<i>Scoparia dulcis</i> L.	Plantaginaceae	D	70	11.57	8.10	5.830	5.380
20	<i>Senna tora</i> (L.) Roxb.	Fabaceae	C	60	5.83	3.50	5.000	2.320
21	<i>Sida acuta</i> Burm.f.	Malvaceae	E	100	22.50	22.50	8.330	14.950
22	<i>Typha angustifolia</i> L.	Typhaceae	C	60	16.16	9.70	5.000	6.440
23	<i>Xenostegia tridentata</i> (L.) D.F.Austn & Staples	Convolvulaceae	B	30	7.67	2.30	2.500	1.520
STATION 3								
1	<i>Alternanthera sessilis</i> (L.) R.Br. ex DC.	Amaranthaceae	D	80	42.13	33.70	6.202	13.184
2	<i>Alternanthera tenella</i> Colla	Amaranthaceae	E	100	38.60	38.60	7.752	15.102
3	<i>Apluda mutica</i> L.	Poaceae	C	60	16.50	9.90	4.651	3.873
4	<i>Boerhaavia diffusa</i> L.	Nyctaginaceae	A	20	4.50	0.90	1.550	0.352
5	<i>Cleome viscosa</i> L.	Cleomaceae	D	70	7.29	5.10	5.426	1.995

6	<i>Corchorus trilocularis</i> L.	Malvaceae	D	70	15.43	10.80	5.426	4.225
7	<i>Cyanthillium cinereum</i> (L.) H.Rob.	Asteraceae	B	30	6.00	1.80	2.326	0.704
8	<i>Cynodon dactylon</i> (L.) Pers.	Poaceae	E	100	29.30	29.30	7.752	11.463
9	<i>Cyperus compressus</i> L.	Cyperaceae	B	40	7.25	2.90	3.101	1.135
10	<i>Cyperus articulatus</i> L.	Cyperaceae	D	70	13.29	9.30	5.426	3.638
11	<i>Cyperus rotundus</i> L.	Cyperaceae	A	20	5.00	1.00	1.550	0.391
12	<i>Dactyloctenium aegyptium</i> (L.) Willd.	Poaceae	D	80	12.38	9.90	6.202	3.873
13	<i>Echinocloa colona</i> (L.) Link.	Poaceae	B	40	17.50	7.00	3.101	2.738
14	<i>Eleusine indica</i> (L.) Gaertn.	Poaceae	D	80	28.00	22.40	6.202	8.763
15	<i>Fimbristylis aestivalis</i> (Retz.) Vahl	Cyperaceae	B	40	9.00	3.60	3.101	1.408
16	<i>Ipomoea aquatica</i> Forssk.	Convolvulaceae	A	10	4.00	0.40	0.775	0.156
17	<i>Mikania micrantha</i> Kunth.	Asteraceae	C	50	25.20	12.60	3.876	4.930
18	<i>Oldenlandia corymbosa</i> L.	Rubiaceae	A	20	1.00	2.00	1.550	0.782
19	<i>Panicum repens</i> L.	Poaceae	C	60	10.00	6.00	4.651	2.347
20	<i>Scoparia dulcis</i> L.	Plantaginaceae	B	40	5.25	2.10	3.101	0.822
21	<i>Sida rhombifolia</i> L.	Malvaceae	E	100	25.40	25.40	7.752	9.937
22	<i>Tridax procumbens</i> L.	Asteraceae	D	70	21.29	14.90	5.426	5.829
23	<i>Trigastrotheca pentaphylla</i> (L.) Thulin	Mollugaceae	B	40	15.00	6.00	3.101	2.347
STATION 4								
1	<i>Aeschynemone indica</i> L.	Fabaceae	B	40	4.00	1.60	4.000	0.994
2	<i>Alternanthera philoxeroides</i> (Mart.) Griseb.	Amaranthaceae	E	100	26.60	26.60	10.000	16.532
3	<i>Alternanthera tenella</i> Colla	Amaranthaceae	E	100	25.50	25.50	10.000	15.848
4	<i>Cleome viscosa</i> L.	Cleomaceae	A	20	3.00	0.60	2.000	0.373
5	<i>Corchorus trilocularis</i> L.	Malvaceae	E	100	20.60	20.60	10.000	12.803
6	<i>Cyanthillium cinereum</i> (L.) H.Rob.	Asteraceae	A	20	1.50	0.30	2.000	0.186
7	<i>Cynodon dactylon</i> (L.) Pers.	Poaceae	E	100	28.40	28.40	10.000	17.651
8	<i>Cyperus rotundus</i> L.	Cyperaceae	C	50	11.20	5.60	5.000	3.480

9	<i>Dactyloctenium aegyptium</i> (L.) Willd.	Poaceae	C	60	15.50	9.30	6.000	5.780
10	<i>Eragrostis viscosa</i> (Retz.) Vahl	Poaceae	E	100	20.60	20.60	10.000	12.803
11	<i>Hibiscus vitifolius</i> L.	Malvaceae	A	10	1.00	0.10	1.000	0.062
12	<i>Ipomoea aquatica</i> Forssk.	Convolvulaceae	A	10	4.00	0.40	1.000	0.248
13	<i>Ludwigia perennis</i> L.	Onagraceae	D	80	6.75	5.40	8.000	3.356
14	<i>Mimosa pudica</i> L.	Fabaceae	D	70	12.86	9.00	7.000	5.594
15	<i>Phyllanthus amarus</i> Schumach. & Thonn.	Phyllanthaceae	A	10	3.00	0.30	1.000	0.186
16	<i>Pseudarthria viscida</i> (L.) Wight & Arn.	Fabaceae	C	50	4.20	2.10	5.000	1.305
17	<i>Scoparia dulcis</i> L.	Plantaginaceae	B	40	7.75	3.10	4.000	1.926
18	<i>Senna tora</i> (L.) Roxb.	Fabaceae	B	40	3.50	1.40	4.000	0.870
STATION 5								
1	<i>Alloteropsis semialata</i> (R.Br.) Hitchc.	Poaceae	E	100	32.20	32.20	8.130	9.990
2	<i>Alternanthera philoxeroides</i> (Mart.) Griseb.	Amaranthaceae	D	70	35.43	24.80	5.691	7.694
3	<i>Alternanthera tenella</i> Colla	Amaranthaceae	E	100	44.60	44.60	8.130	13.838
4	<i>Ammannia baccifera</i> L.	Lythraceae	D	70	17.00	11.90	5.691	3.692
5	<i>Blumea oxyodonta</i> DC.	Asteraceae	E	100	6.30	6.30	8.130	1.955
6	<i>Cynodon dactylon</i> (L.) Pers.	Poaceae	E	100	35.70	35.70	8.130	11.076
7	<i>Eleusine indica</i> (L.) Gaertn.	Poaceae	E	100	22.80	22.80	8.130	7.074
8	<i>Hygrophila auriculata</i> (K.Schum) Heine	Acanthaceae	D	70	32.14	22.50	5.691	6.981
9	<i>Mesosphaerum sauveolens</i> (L.) Kuntze	Lamiaceae	D	70	8.14	5.70	5.691	1.768
10	<i>Mimosa pudica</i> L.	Fabaceae	E	100	38.30	38.30	8.130	11.883
11	<i>Senna tora</i> (L.) Roxb.	Fabaceae	C	50	9.00	4.50	4.065	1.396
12	<i>Sida acuta</i> Burm.f.	Malvaceae	E	100	41.00	41.00	8.130	12.721
13	<i>Sphaeranthus indicus</i> L.	Asteraceae	D	80	18.75	15.00	6.504	4.654
14	<i>Sphagneticola trilobata</i> (L.) Pruski	Asteraceae	A	20	6.50	1.30	1.626	0.403
15	<i>Urena lobata</i> L.	Malvaceae	E	100	15.70	15.70	8.130	4.871

STATION 6								
1	<i>Abutilon indicum</i> (Link) Sweet.	Malvaceae	B	30	6.33	1.90	1.456	0.552
2	<i>Achyranthes aspera</i> L.	Amaranthaceae	B	30	13.33	4.00	1.456	1.164
3	<i>Ageratum conyzoides</i> L.	Asteraceae	C	50	9.00	4.50	2.427	1.309
4	<i>Alternanthera tenella</i> Colla	Amaranthaceae	D	80	22.25	17.80	3.883	5.180
5	<i>Alysicarpus vaginalis</i> (L.) DC.	Fabaceae	E	100	18.20	18.20	4.854	5.296
6	<i>Amaranthus spinosus</i> L.	Amaranthaceae	A	10	4.00	0.40	0.485	0.116
7	<i>Asystasia gangetica</i> (L.) T.Anderson	Acanthaceae	D	70	19.86	13.90	3.398	4.045
8	<i>Bacopa monnieri</i> (L.) Wettst.	Plantaginaceae	A	20	8.00	1.60	0.970	0.465
9	<i>Blumea oxyodonta</i> DC.	Asteraceae	A	20	10.50	2.10	0.970	0.611
10	<i>Cardiospermum halicacabum</i> L.	Sapindaceae	A	20	2.00	0.40	0.970	0.116
11	<i>Chromolaena odorata</i> (L.) R.M.King & H.Rob.	Asteraceae	C	50	21.00	10.10	2.427	2.939
12	<i>Cleome viscosa</i> L.	Cleomaceae	A	20	3.00	0.60	0.970	0.174
13	<i>Colocasia esculenta</i> (L.) Scholt.	Araceae	A	10	5.00	0.50	0.485	0.145
14	<i>Commelina benghalensis</i> L.	Commelinaceae	A	20	15.50	3.10	0.970	0.902
15	<i>Corchorus trilocularis</i> L.	Malvaceae	E	100	20.40	20.40	4.854	5.937
16	<i>Cynodon dactylon</i> (L.) Pers.	Poaceae	E	100	28.60	28.60	4.854	8.323
17	<i>Cyperus articulatus</i> L.	Cyperaceae	D	70	12.43	8.70	3.398	2.532
18	<i>Cyperus brevifolius</i> (Rottb.) Hassk.	Poaceae	D	80	20.13	16.10	3.883	4.685
19	<i>Eleusine indica</i> (L.) Gaertn.	Poaceae	E	100	371.00	37.10	4.854	10.797
20	<i>Hibiscus vitifolius</i> L.	Malvaceae	B	40	1.75	0.70	1.942	0.203
21	<i>Hygrophila auriculata</i> (K.Schum) Heine	Acanthaceae	E	100	13.80	13.80	4.854	4.016
22	<i>Jatropha gossypifolia</i> L.	Euphorbiaceae	A	10	1.00	0.10	0.485	0.029
23	<i>Ludwigia perennis</i> L.	Onagraceae	C	50	8.40	4.20	2.427	1.222
24	<i>Mikania micrantha</i> Kunth.	Asteraceae	A	10	3.00	0.30	0.485	0.087
25	<i>Mimosa pudica</i> L.	Fabaceae	E	100	26.80	26.80	4.854	7.799
26	<i>Panicum notatum</i> Retz.	Poaceae	D	70	9.43	6.60	3.398	1.920

27	<i>Persicaria barbata</i> (L.) H.Hara	Polygonaceae	D	80	14.00	11.20	3.883	3.259
28	<i>Pontederia crassipes</i> Mart.	Pontederiaceae	A	10	13.00	1.30	0.485	0.378
29	<i>Pseudarthria viscida</i> (L.) Wight & Arn.	Fabaceae	A	20	2.50	0.50	0.970	0.145
30	<i>Scoparia dulcis</i> L.	Plantaginaceae	D	80	8.75	7.00	3.883	2.037
31	<i>Senna tora</i> (L.) Roxb.	Fabaceae	C	60	9.67	5.80	2.912	1.688
32	<i>Sida acuta</i> Burm.f.	Malvaceae	D	80	31.25	25.00	3.883	7.275
33	<i>Solanum torvum</i> Sw.	Solanaceae	B	40	6.25	2.50	1.942	0.727
34	<i>Sphaeranthus indicus</i> L.	Asteraceae	E	100	10.50	10.50	4.854	0.305
35	<i>Sphagneticola trilobata</i> (L.) Pruski	Asteraceae	A	20	3.00	0.60	0.970	0.174
36	<i>Synedrella nodiflora</i> (L.) Gaertn.	Asteraceae	E	90	12.00	10.80	4.369	3.143
37	<i>Triumfetta rhomboidea</i> Jacq.	Malvaceae	B	40	7.25	2.90	1.942	0.844
38	<i>Urena lobata</i> L.	Malvaceae	D	80	28.75	23.00	3.883	6.693
STATION 7								
1	<i>Aerva lanata</i> (L.) Juss. ex Schult.	Amaranthaceae	B	20	3.00	0.60	1.769	0.335
2	<i>Alternanthera philoxeroides</i> (Mart.) Griseb.	Amaranthaceae	E	100	24.70	24.70	8.849	13.806
3	<i>Alternanthera tenella</i> Colla	Amaranthaceae	E	100	35.30	35.30	8.849	19.731
4	<i>Amaranthus spinosus</i> L.	Amaranthaceae	B	40	3.25	1.30	3.539	0.726
5	<i>Ammannia baccifera</i> L.	Lythraceae	E	100	18.70	18.70	8.849	10.453
6	<i>Boerhavia diffusa</i> L.	Nyctaginaceae	A	20	2.50	0.50	1.769	0.279
7	<i>Cleome viscosa</i> L.	Cleomaceae	C	60	2.67	1.60	5.309	0.894
8	<i>Croton bonplandianus</i> Baill.	Euphorbiaceae	E	100	14.20	14.20	8.849	7.937
9	<i>Cynodon dactylon</i> (L.) Pers.	Poaceae	E	100	7.40	7.40	8.849	4.136
10	<i>Cyperus articulatus</i> L.	Cyperaceae	A	20	2.50	0.50	1.769	0.279
11	<i>Cyperus rotundus</i> L.	Cyperaceae	D	70	3.43	2.40	6.194	1.342
12	<i>Heliotropium indicum</i> L.	Boraginaceae	B	40	2.50	1.00	3.539	0.559
13	<i>Ipomoea triloba</i> L.	Convolvulaceae	B	40	2.50	1.00	3.539	0.559
14	<i>Persicaria barbata</i> (L.) H.Hara	Polygonaceae	E	100	43.70	43.70	8.849	24.427

15	<i>Pontederia crassipes</i> Mart.	Pontederiaceae	A	20	5.50	1.10	1.769	0.614
16	<i>Ricinus communis</i> L.	Euphorbiaceae	D	80	14.63	11.70	7.079	6.540
17	<i>Senna tora</i> (L.) Roxb.	Fabaceae	B	40	3.75	1.50	3.539	0.838
18	<i>Sphaeranthus indicus</i> L.	Asteraceae	C	60	18.67	11.20	5.309	6.260
19	<i>Xanthium strumarium</i> L.	Asteraceae	A	20	2.50	0.50	1.769	0.279
STATION 8								
1	<i>Alternanthera philoxeroides</i> (Mart.) Griseb.	Amaranthaceae	E	100	36.10	36.10	6.410	15.931
2	<i>Alternanthera tenella</i> Colla	Amaranthaceae	E	100	44.70	44.70	6.410	19.726
3	<i>Amaranthus spinosus</i> L.	Amaranthaceae	A	10	5.00	0.50	0.641	0.220
4	<i>Ammannia baccifera</i> L.	Lythraceae	C	60	12.33	7.40	3.846	3.265
5	<i>Calotropis gigantea</i> (L.) W.T.Aiton	Apocynaceae	B	30	2.00	0.60	1.923	0.264
6	<i>Cleome viscosa</i> L.	Cleomaceae	C	50	3.60	1.80	3.205	0.794
7	<i>Coccinia indica</i> L.	Cucurbitaceae	A	20	2.50	0.50	1.282	0.220
8	<i>Colocasia esculenta</i> (L.) Scholt.	Araceae	B	30	5.33	1.60	1.923	0.706
9	<i>Commelina benghalensis</i> L.	Commelinaceae	E	90	3.11	2.80	5.769	1.235
10	<i>Croton bonplandianus</i> Baill.	Euphorbiaceae	D	80	22.63	18.10	5.128	7.987
11	<i>Cucumis melo</i> L.	Cucurbitaceae	A	20	2.00	0.40	1.282	0.176
12	<i>Cynodon dactylon</i> (L.) Pers.	Poaceae	D	70	12.29	8.60	4.487	3.795
13	<i>Cyperus rotundus</i> L.	Cyperaceae	E	100	4.90	4.90	6.410	2.162
14	<i>Echinocloa colona</i> (L.) Link	Poaceae	E	100	22.70	22.70	6.410	10.017
15	<i>Eleusine indica</i> (L.) Gaertn.	Poaceae	D	70	18.57	13.00	4.487	5.736
16	<i>Heliotropium indicum</i> L.	Boraginaceae	B	30	4.00	1.20	1.923	0.529
17	<i>Lycopersicon esculentum</i> L.	Solanaceae	A	10	2.00	0.20	0.641	0.088
18	<i>Mimosa pudica</i> L.	Fabaceae	E	100	7.50	7.50	6.410	3.309
19	<i>Oldenlandia corymbosa</i> L.	Rubiaceae	B	30	2.00	0.60	1.923	0.264
20	<i>Parthenium hysterophorus</i> L.	Asteraceae	B	30	5.33	1.60	1.923	0.706
21	<i>Persicaria barbata</i> (L.) H.Hara	Polygonaceae	D	70	24.29	17.00	4.487	7.502

22	<i>Persicaria glabra</i> (Willd.)M.Gomez	Polygonaceae	B	40	28.00	11.20	2.564	4.942
23	<i>Physalis minima</i> L.	Solanaceae	B	30	3.00	0.90	1.923	0.397
24	<i>Ricinus communis</i> L.	Euphorbiaceae	C	60	2.83	1.70	3.846	0.750
25	<i>Saccharum spontaneum</i> L.	Poaceae	B	40	14.25	5.70	2.564	2.515
26	<i>Scoparia dulcis</i> L.	Plantaginaceae	C	50	7.80	3.90	3.205	1.721
27	<i>Senna tora</i> (L.) Roxb.	Fabaceae	C	50	5.60	2.80	3.205	1.235
28	<i>Trianthema portulacastrum</i> L.	Aizoaceae	A	20	11.50	2.30	1.282	1.015
29	<i>Trigastrotheca pentaphylla</i> (L.) Thulin	Mollugaceae	C	50	9.00	4.50	3.205	1.985
30	<i>Urena lobata</i> L.	Malvaceae	A	20	9.00	1.80	1.282	0.794
STATION 9								
1	<i>Alternanthera sessilis</i> (L.) R.Br. ex DC.	Amaranthaceae	E	100	25.80	25.80	4.386	6.445
2	<i>Alternanthera tenella</i> Colla	Amaranthaceae	E	100	40.60	40.60	4.386	10.142
3	<i>Alysicarpus vaginalis</i> (L.) DC.	Fabaceae	B	40	8.50	3.40	1.754	0.849
4	<i>Ammannia baccifera</i> L.	Lythraceae	D	80	14.63	11.70	3.509	2.922
5	<i>Asystasia gangetica</i> (L.) T. Anderson	Acanthaceae	D	70	24.57	17.20	3.070	0.429
6	<i>Cenchrus polystachios</i> (L.) Morrone	Poaceae	D	80	10.75	8.60	3.509	2.148
7	<i>Chromolaena odorata</i> (L.) R.M.King & H.Rob.	Asteraceae	C	60	14.33	8.60	2.632	2.148
8	<i>Commelina benghalensis</i> L.	Commelinaceae	B	30	16.67	5.00	1.316	1.249
9	<i>Corchorus trilocularis</i> L.	Malvaceae	E	90	19.11	17.90	3.947	4.471
10	<i>Cyanotis axillaris</i> (L.) D. Don ex Sweet	Commelinaceae	B	40	5.75	2.30	1.754	0.574
11	<i>Cynodon dactylon</i> (L.) Pers.	Poaceae	E	100	16.60	16.60	4.386	4.146
12	<i>Cyperus compressus</i> L.	Cyperaceae	D	70	10.29	7.20	3.070	1.798
13	<i>Cyperus mindorensis</i> (Steud.) Huygh	Cyperaceae	D	80	25.75	20.60	3.509	5.146
14	<i>Cyperus rotundus</i> L.	Cyperaceae	C	50	8.20	4.10	2.193	1.024
15	<i>Dactyloctenium aegyptium</i> (L.) Willd.	Poaceae	D	80	7.38	5.90	3.509	1.473
16	<i>Echinochloa colona</i> (L.) Link.	Poaceae	E	100	26.60	26.60	4.386	6.645
17	<i>Eleusine indica</i> (L.) Gaertn.	Poaceae	C	60	13.67	8.20	2.632	2.048

18	<i>Eragrostis viscosa</i> (Retz.) Trin.	Poaceae	E	100	21.80	21.80	4.386	5.445
19	<i>Grona heterophylla</i> (Willd.) H.Ohashi & K. Ohashi	Fabaceae	D	80	14.13	11.30	3.508	2.822
20	<i>Ludwigia perennis</i> L.	Onagraceae	C	60	7.17	4.30	2.632	1.074
21	<i>Mimosa pudica</i> L.	Fabaceae	E	100	37.60	37.60	4.386	9.392
22	<i>Oldenlandia corymbosa</i> L.	Rubiaceae	B	30	3.67	1.10	1.316	0.274
23	<i>Panicum notatum</i> Retz.	Poaceae	C	60	11.83	7.10	2.632	1.773
24	<i>Physalis minima</i> L.	Solanaceae	B	30	3.00	0.90	1.316	0.224
25	<i>Pseudarthria viscida</i> (L.) Wight & Arn.	Fabaceae	B	40	3.75	1.50	1.754	0.374
26	<i>Scoparia dulcis</i> L.	Plantaginaceae	D	80	6.25	5.00	3.508	1.249
27	<i>Senna tora</i> (L.) Roxb.	Fabaceae	D	70	10.29	7.20	3.070	1.798
28	<i>Sida acuta</i> Burm.f.	Malvaceae	D	80	24.13	1.30	3.509	4.821
29	<i>Spermacoce hispida</i> L.	Rubiaceae	B	40	2.75	1.10	1.754	0.274
30	<i>Sphaeranthus indicus</i> L.	Asteraceae	D	80	14.75	11.80	3.509	2.947
31	<i>Synedrella nodiflora</i> (L.) Gaertn.	Asteraceae	D	70	9.57	6.70	3.070	1.673
32	<i>Triumfetta rhomboidea</i> Jacq.	Malvaceae	B	30	7.33	2.20	1.316	0.549
33	<i>Urena lobata</i> L.	Malvaceae	E	100	31.10	31.10	4.386	7.769
STATION 10								
1	<i>Abelmoschus angulosus</i> Wall. ex Wight & Arn.	Malvaceae	A	10	5.00	0.50	0.595	0.237
2	<i>Alternanthera philoxeroides</i> (Mart.) Griseb.	Amaranthaceae	E	100	28.30	28.30	5.952	13.463
3	<i>Alternanthera tenella</i> Colla	Amaranthaceae	E	100	27.10	27.10	5.952	12.892
4	<i>Amaranthus spinosus</i> L.	Amaranthaceae	A	20	3.00	0.60	1.190	0.285
5	<i>Ammannia baccifera</i> L.	Lythraceae	C	50	11.00	5.50	2.976	2.616
6	<i>Calotropis gigantea</i> (L.) W.T.Aiton	Apocynaceae	A	20	2.00	0.40	1.190	0.190
7	<i>Chromolaena odorata</i> (L.) R.M.King & H.Rob.	Asteraceae	C	60	7.17	4.30	3.571	2.045
8	<i>Cleome viscosa</i> L.	Cleomaceae	B	40	4.25	1.70	2.381	0.808
9	<i>Colocasia esculenta</i> (L.) Scholt.	Araceae	A	20	2.00	0.40	1.190	0.190
10	<i>Corchorus trilocularis</i> L.	Malvaceae	C	50	8.80	4.40	2.976	2.093

11	<i>Cucumis melo</i> L.	Cucurbitaceae	A	20	5.00	1.00	1.190	0.475
12	<i>Cynodon dactylon</i> (L.) Pers.	Poaceae	E	100	19.90	19.90	5.952	9.467
13	<i>Cyperus compressus</i> L.	Cyperaceae	C	60	5.00	3.00	3.571	1.427
14	<i>Cyperus articulatus</i> L.	Cyperaceae	C	60	7.67	4.60	3.571	2.188
15	<i>Dactyloctenium aegyptium</i> (L.) Willd.	Poaceae	C	50	13.60	6.80	2.976	3.235
16	<i>Eragrostis unioides</i> (Retz.) Nees ex Steud.	poaceae	D	70	12.57	8.80	4.166	4.186
17	<i>Hygrophila auriculata</i> (K.Schum) Heine	Acanthaceae	C	60	23.50	14.10	3.571	6.707
18	<i>Ipomoea aquatica</i> Forssk.	Convolvulaceae	A	20	3.00	0.60	1.190	0.285
19	<i>Lantana camara</i> L.	Verbenaceae	A	20	5.50	1.10	1.190	0.523
20	<i>Ludwigia perennis</i> L.	Onagraceae	C	60	3.33	2.00	3.571	0.951
21	<i>Melochia corchorifolia</i> L.	Malvaceae	B	30	2.67	0.80	1.786	0.380
22	<i>Mikania micrantha</i> Kunth.	Asteraceae	B	30	6.33	1.90	1.786	0.903
23	<i>Mimosa diplotricha</i> C. Wright	Fabaceae	D	70	20.86	14.60	4.166	6.945
24	<i>Mimosa pudica</i> L.	Fabaceae	B	30	7.33	2.20	1.786	1.046
25	<i>Oldenlandia corymbosa</i> L.	Rubiaceae	B	40	3.75	1.50	2.381	0.713
26	<i>Panicum repens</i> L.	Poaceae	D	70	13.71	9.60	4.166	4.567
27	<i>Pontederia crassipes</i> Mart.	Pontederiaceae	A	20	3.00	0.60	1.190	0.285
28	<i>Scoparia dulcis</i> L.	Plantaginaceae	C	50	3.80	1.90	2.976	0.903
29	<i>Sida acuta</i> Burm.f.	Malvaceae	E	100	18.00	18.00	5.952	8.563
30	<i>Sphaeranthus indicus</i> L.	Asteraceae	C	50	20.00	10.00	2.976	4.757
31	<i>Stachytarpheta jamaicensis</i> (L.) Vahl.	Verbenaceae	B	40	7.00	2.80	2.381	1.332
32	<i>Stemodia viscosa</i> Roxb.	Plantaginaceae	A	20	13.50	2.70	1.190	1.284
33	<i>Tridax procumbens</i> L.	Asteraceae	B	40	7.75	3.10	2.381	1.474
34	<i>Trigastrotheca pentaphylla</i> (L.) Thulin	Molluginaceae	A	20	3.00	0.60	1.190	0.285
35	<i>Triumfetta rhomboidea</i> Jacq.	Malvaceae	B	30	5.67	1.70	1.786	0.808
36	<i>Urena lobata</i> L.	Malvaceae	C	50	6.20	3.10	2.976	1.474

(F –frequency, D – density, A – abundance, IVI – Important Value Index, A/F – abundance / frequency).

The analysis of the vegetation showed that 54.6% of the recorded flora in the study area belonged to just six major families (Fig. 2.3). Poaceae was the most speciose herb family with 12 species under 10 genera with a high density of *Cynodon dactylon* attributed towards it. The diversified family Asteraceae (11 species) was co-dominant which also contributed to the species richness. The flora exhibited a high degree of monotypism, with nearly half of the recorded families (40.6%) represented by only one species. A total of thirteen life-forms was identified. Therophytes were the most abundant accounting for 39 species, followed by Therophytes-Chamaephytes with 25 species. Hydrophytes were least represented with a single species (*Pontederia crassipes* Mart.) (Fig. 2.4). Most of the stations showed high similarity (positive correlation). A high similarity was observed between the stations 7 and 8 ($r = 0.73$), stations 5 and 10 ($r = 0.63$), and stations 5 and 6 ($r = 0.59$). However, for the stations with low similarity, the correlation was not significant (Table 2.2).

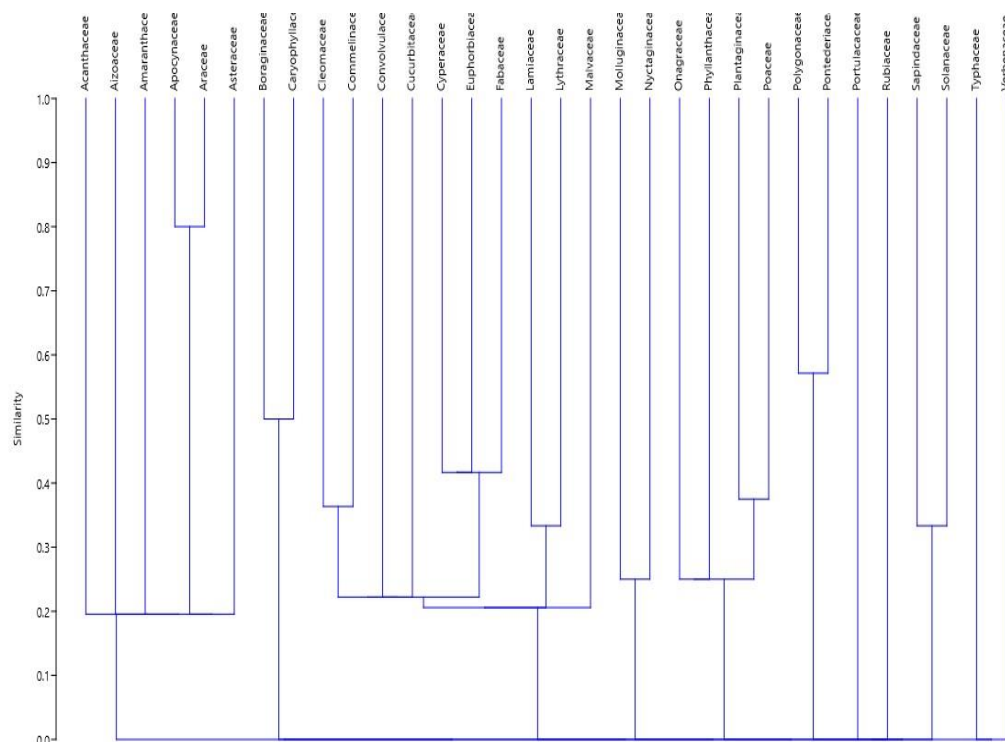


Fig. 2.3. Hierarchical clustering dendrogram representing the floristic similarity among plant families in the study area. Higher values indicate greater resemblance between families in terms of species composition.

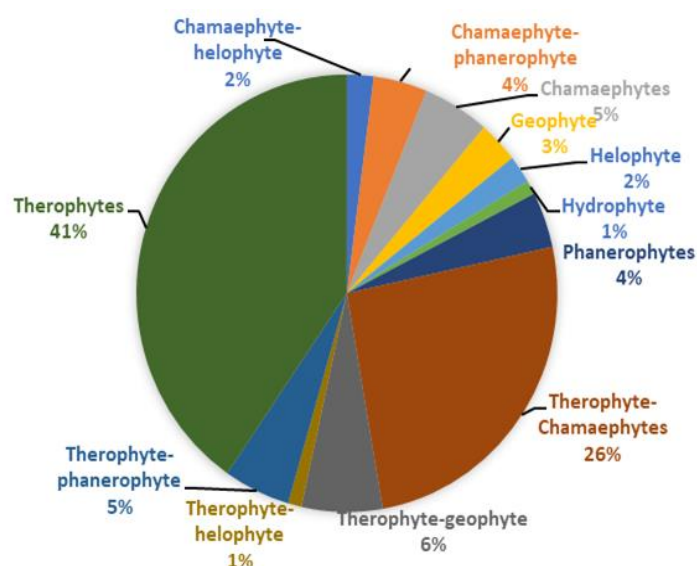


Fig. 2.4. Life form spectrum showing the distribution of different plant categories documented in the study area.

Table 2.2. Pearson correlation between plant species richness in the study sites.

	STATION 1	STATION 2	STATION 3	STATION 4	STATION 5	STATION 6	STATION 7	STATION 8	STATION 9	STATION 10
STATION 1	1.000**									
STATION 2	0.013	1.000**								
STATION 3	0.078	0.426**	1.000**							
STATION 4	-0.108066	0.282**	0.414**	1.000**						
STATION 5	-0.063586	0.550**	0.273**	0.455**	1.000**					
STATION 6	0.008	0.371**	0.251*	0.321**	0.599**	1.000**				
STATION 7	0.046	0.076	0.202*	0.393**	0.377**	0.175	1.000**			
STATION 8	0.204*	0.157	0.359**	0.507**	0.484**	0.257*	0.731**	1.000**		
STATION 9	-0.066688	0.314**	0.391**	0.469**	0.508**	0.551**	0.184	0.373**	1.000**	
STATION 10	-0.129052	0.415**	0.323**	0.562**	0.631**	0.359**	0.430**	0.432**	0.335**	1.000**

The correlation coefficients range from -1 to 1 where positive values shows a direct relationship and negative values indicate an inverse relationship.

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

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

In the study, maximum plants were observed at sampling station 6 followed by stations 10 and 9, while minimum plant species were recorded at station 5. Frequency measures the commonness and distribution of a species within a study area (Ghavzan *et al.*, 2006). The species which have maximum frequency index included *A. tenella*, *A. sessilis*, *Sida acuta*, *Corchorus trilocularis*, *Mimosa pudica* and *Cynodon dactylon*. *Stemodia viscosa*, *Abelmoschus angulosus*, *Jatropha gossypifolia*, *Luffa*

cylindrica were having the lowest percentage frequency index. The vegetation represented heterogeneous nature at all sampling stations based on Raunkiaer's frequency classes and was observed to be high in more polluted areas. The Raunkiaer's frequency law ($A > B > C \geq \text{or} \leq D < E$) was found to be highly disrupted in all the sampling stations which implies that the community is heterogeneous, and some species are dominant over the others.

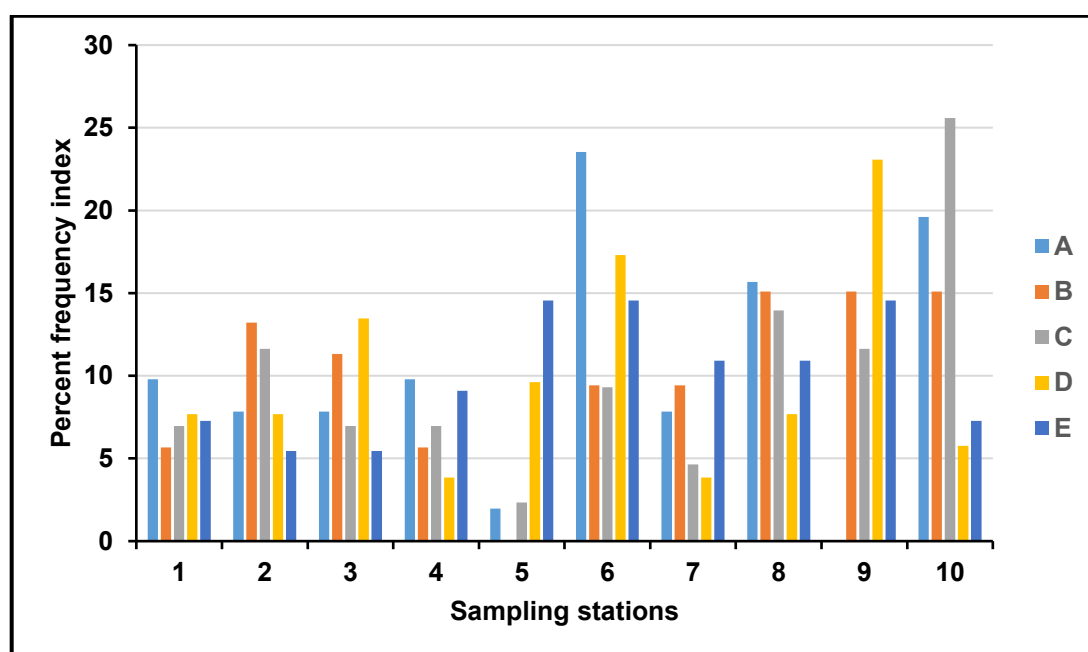


Fig. 2.5. Raunkiaer's frequency classes in the study area. The observed pattern deviates from the expected frequency rule ($A > B > C \geq D < E$) indicating irregular species distribution patterns across sampling stations and potential ecological influences.

The graphic representation of Raunkiaer's frequency classes for different sampling stations is given in Fig. 2.5. An attempt to understand the human impact on species diversity and its pattern has been made in the present study. Based on quantitative analysis, overall species richness across stations follows the rank order station-6 > station-10 > station-9 > station-8 > station-3 = station-2 > station-7 = station-1 > station-4 > station-5. Fig. 2.6 presents the Important Value Index (IVI) of the ten dominant plant species in the study area. In the study, the IVI estimates indicated the dominance of weeds like *A. tenella*, *C. dactylon*, *S. acuta* and *P. barbata* in most of the quadrats studied.

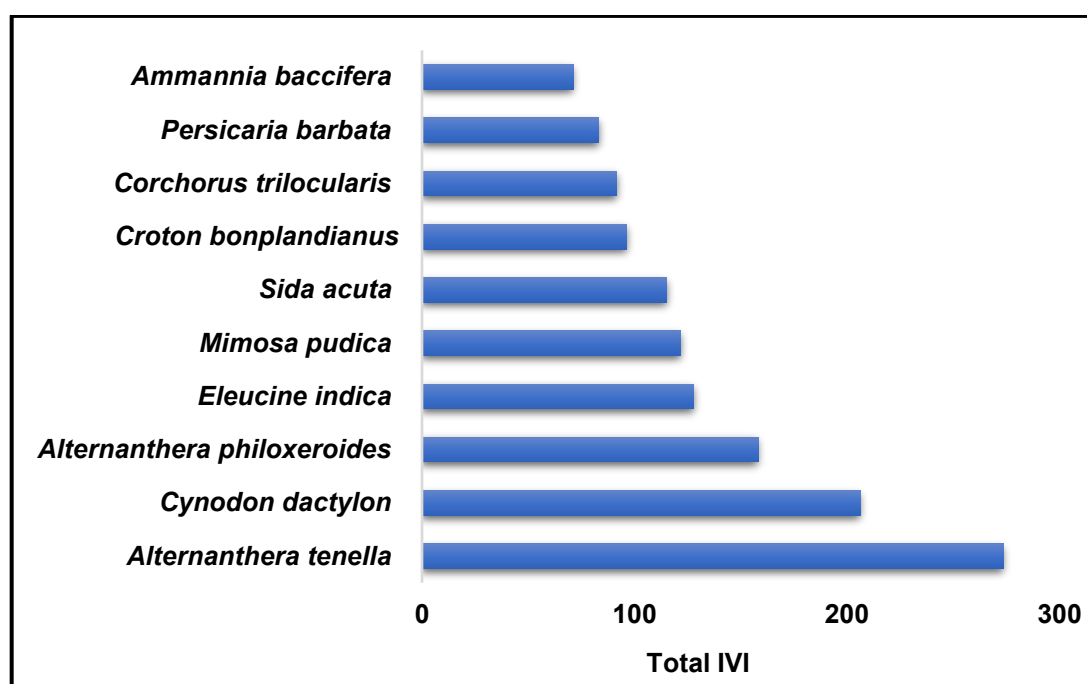


Fig. 2.6. Graph showing top ten plant species in the study area based on their IVI values.

Table 2.3. Different diversity indices calculated for the sampling stations

Plot	1	2	3	4	5	6	7	8	9	10
Cd	0.892	0.913	0.910	0.874	0.906	0.945	0.855	0.902	0.947	0.929
H'	2.484	2.653	2.621	2.262	2.475	3.11	2.192	2.688	3.143	2.971
e	0.631	0.645	0.625	0.534	0.792	0.590	0.471	0.490	0.702	0.542
Dmn	0.493	0.582	0.442	0.449	0.264	0.648	0.449	0.630	0.522	0.785
SR	2.465	2.89	2.687	2.302	1.733	4.544	2.403	3.754	3.858	4.575
d	0.230	0.157	0.156	0.177	0.138	0.108	0.244	0.197	0.101	0.135

(Cd – Simpson's index of dominance, H' – Shannon-Wiener index, e – Pielou's evenness index, Dmn – Menhinick index, SR – Margalef's index, d – Berger-Parker index).

Six different diversity indices were used to analyse the herbaceous vegetation in all the sampling stations (Table 2.3). All stations showed higher values for the diversity indices which shows that the vegetation in the study area falls within the

range of diverse natural communities. The values of species diversity (H') ranged between 2.192 to 3.143 and Simpson's dominance index between 0.855 and 0.947 indicated that the diversity of riparian vegetation was within the range of diverse natural communities. The species are distributed rather evenly since Shannon-Wiener and Simpson's diversity indices in between various stations do not show much significant differences. The species evenness ranged from 0.471 to 0.792. The Berger-Parker index varied from 0.101 to 0.244. Margalef's species richness index (SR) ranged from 1.733 to 4.575 and Menhinick's index varied from 0.264 to 0.785. The indices that represent the evenness of distribution appeared to be fluctuating within the stations which indicates that some species appeared in thickets; they were not distributed uniformly.

The structural and functional community organization is provided by the Dominance-distribution (DD) curve. The D-D curve based on the Importance Value Index of herb species in the study area reveals a log-normal distribution pattern as shown in Fig. 2.7. A reverse J-shaped D-D curve was observed for herb species which indicates that most species exhibit low dominance. The D-D curve exhibited a log-normal distribution pattern (Preston, 1948) and indicated high species richness in low dominance (IVI range) which agreed with the observation of Whittaker (1975). The abundance to frequency ratio (A/F) indicated contagious distribution for the majority of species in the study area, with regular distribution being rare among plants. The herb species *Cleome viscosa*, *Commelina benghalensis*, and *Cyperus rotundus* were found to be the only plants that showed a regular distribution pattern in the studied sites. Species like *Coldenia procumbens*, *Glinus oppositifolius*, *Sida rhombifolia* etc. were restricted in distribution but were abundant locally. Plants like *A. angulosus*, *Portulaca oleracea*, *Coccinia grandis*, etc. exhibited restricted distribution and were rare in occurrence. The species with a wide distribution range and high abundance in riverbank included *A. tenella*, *A. sessilis*, *S. acuta*, *M. pudica* and *C. dactylon*.

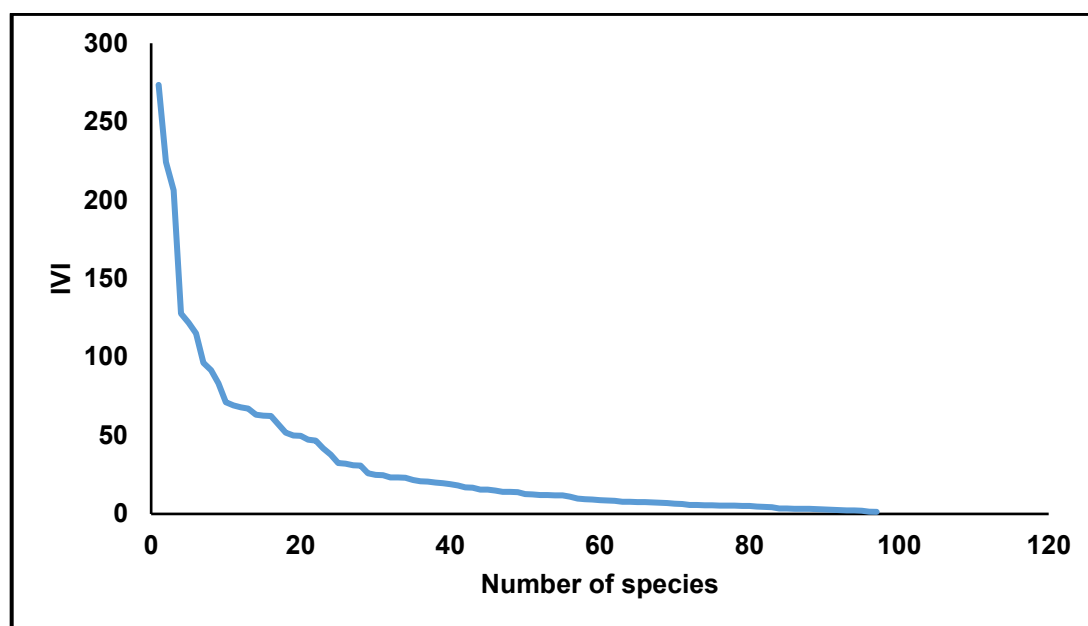


Fig. 2.7. Dominance-distribution curve of the herb species based on their IVI within the study area.

2.4.2. Heavy metal accumulation in plant and soil samples

In the phytosociological study, *A. tenella* Colla was observed to be the dominant plant species. Concentrations of heavy metals, including Cr, Mn, Zn, Cu, Ni, Pb, Cd, and Co, were assessed using ICP-AES in both *A. tenella* and soil samples collected from the study area. Although all the analyzed heavy metals were present in the samples, only Cr, Mn, and Zn were found in higher concentrations, as indicated in Table 2.4. The study further provides BAC, BTC, and BCF values for various heavy metals in *A. tenella*, offering insights into its metal accumulation characteristics. The quantification of heavy metals revealed the accumulation of Cr, Mn, and Zn in various plant parts and soil. Pb, Ni, Cd, Cu, and Co were also present in the plant samples but in low quantities. The soil sample collected from the study area contained Cr (155.09 mgkg^{-1}), Mn (44.60 mgkg^{-1}), and Zn (48.38 mgkg^{-1}). The maximum concentration of Cr and Mn in the plant sample was observed in the roots ($26.2375 \text{ mgkg}^{-1}$ and 5.4875 mgkg^{-1} respectively), but for Zn, the total concentration in plant samples was highest in the leaf (63.44 mgkg^{-1}). *A. tenella* exhibited significant values exceeding 1 for both BAC and BTC for Zn, indicating its high potential for translocating and accumulating Zn in its shoots.

Table 2.4. Concentrations of different heavy metals in plant and soil samples and various coefficients

Heavy metal	Soil (mgkg ⁻¹)	Root (mgkg ⁻¹)	Shoot (mgkg ⁻¹)	BAC	BTC	BCF
Cr	155.09	26.238	19.831	0.1279	0.7558	0.1692
Mn	44.60	5.486	3.348	0.0751	0.6101	0.1230
Zn	48.38	18.825	85.103	1.7590	4.5207	0.3891

(BAC – biological accumulation coefficient, BTC – biological transfer coefficient, BCF – bioconcentration factor).

2.5. Discussion

2.5.1. Floristic composition

A plant community refers to the grouping of diverse plant species coexisting in a specific habitat. Analyzing plant communities is crucial for comprehending the functional characteristics and vegetation structure of a given area (Boruah *et al.*, 2020). In terms of the floristic diversity, 97 species belonging to 83 genera and 32 families were recorded from the study area. The result indicates the high density and diversity of species in this riparian area, which is comparable to the species richness reported in other riparian areas affected by irrational anthropogenic activities (Singhal *et al.*, 2016; Nazir *et al.*, 2011; Sharma *et al.*, 2016). Poaceae and Asteraceae had the widest distribution in the study area which is like the observations of Singhal *et al.* (2016) and Mohanty *et al.* (2020) along the polluted riverbanks. This distribution is due to efficient dispersal, successful establishment, ecological adaptability, disturbance tolerance, and the ability to modify ecosystems through mammalian herbivory dynamics (Abbas *et al.*, 2021).

Poaceae was the dominant family in several studies on the species distribution and richness of pollution tolerant plants around the industries (Noukeu *et al.*, 2019; Anoliefo *et al.*, 2008). The dominance of Poaceae members may also result from their effective utilization of soil nitrogen in contaminated ecosystems, providing them with a competitive edge over other plant families (Zhang *et al.*, 2020). The high level of monotypism found in the study area could be attributed to the limited

number of plants capable of withstanding the harsh conditions caused by industrial effluent discharge. The life form spectrum of the Kanjikode industrial area was dominated by therophytes (41%), followed by Therophytes-Chamaephytes (25.8%). The prevalence of therophytes can be due to their short life cycle, rapid growth, genetic adaptability, and resilience to various disturbances like water fluctuations, hot climates, topographic variations, biotic influences, and human activities (Abbas *et al.*, 2021).

In ecological studies, IVI indicates the ecological significance of a specific species within an ecosystem and success of the corresponding species in an ecosystem (Odum, 1971; Asigbaase *et al.*, 2019). The maximum numbers of species that predominate the vegetation in industrially contaminated riparian zones exhibit rapid growth and possess extensive root systems, facilitating broad surface coverage and deep penetration into the contaminated soil. The higher IVI value for herbaceous plants may result from their enhanced tolerance to industrial contaminants, characterized by sufficient biomass formation, reproductive capability under pollution stress, appropriate structure, and resilience to long-term pollutant impacts. Invasive alien plants like *Ageratum conyzoides*, *A. philoxeroides*, *Pontederia crassipes*, *Ipomoea cairica*, *Mikania micrantha*, *Mimosa diplotricha*, *Sphagneticola trilobata*, etc. were found to be proliferating rapidly in a study conducted by KCSTE - Malabar Botanical Garden and Institute for Plant Sciences (2019) for Kerala Biodiversity Board on the impact of floods on riparian biodiversity. This proliferation threatens the indigenous flora of the flood-affected river basins. The results of the present study reflect the same observation with high IVI values for such invasive plants. Despite the resource scarcity and pollutants in soil (Sajeesh *et al.*, 2015), the vegetation is highly diversified along Walayar Riverbank which implies that those plants can be used for phytoremediation ability and for maintaining green cover in disturbed areas (Singhal *et al.*, 2016). The Whitford index or ratio of abundance to frequency (A/F) serves as an indicator for understanding the distribution pattern of species within a community. The findings of the present study align with those of Boruah *et al.* (2020), Shameem *et al.* (2010), and Shadangi and Nath (2005), all indicating that the distribution of most plant

species in natural vegetation tends to be contagious thus supporting the outcomes of the present study. The contiguous distribution prevalent in the study area is attributed to reproductive strategies of herbaceous plant species, where most species reproduce vegetatively in addition to sexual reproduction. These species eliminate competitors by means of rapid growth and prolific offspring production. This pattern aligns with suggestions that contiguous distribution is common in areas with notable environmental variation, as proposed by Odum (1971) and observed by Alhassan *et al.* (2006).

2.5.2. Heavy metal accumulation in plant and soil samples

Various studies have reported the presence of heavy metal in soil or sediments near industrial rivers such as Hoque *et al.* (2023), Gupta *et al.* (2023), Sojka and Jaskuła (2022), Moldovan *et al.* (2022), Ipeaiyeda and Onianwa (2018), Sabo and Ladan (2018), and Ratheesh-Chandra *et al.* (2018). In this study, Cr concentration was higher in the root of the plant sample, aligning with findings by Mohanty *et al.* (2020) and Homa *et al.* (2016), indicating that plants accumulate higher levels of Cr in the roots than in aerial parts. This is attributed to the phytostabilization process, as described by Padmavathiamma and Li (2007). The maximum concentration of Mn in the root is consistent with high Mn concentrations reported in species such as *Salix*, *Typha*, and *Arundo* along the Lana River (Shehu *et al.*, 2010). The higher concentration of Zn in the leaves indicates the high mobility of Zn from roots to shoots through the phytoextraction process (Cheng, 2003). Several studies have reported the accumulation of heavy metals in plant roots than the aerial parts (Yan *et al.*, 2020; Singh *et al.*, 2021; Ahmad *et al.*, 2020; Zong *et al.*, 2020). This is due to their limited mobility compared to essential nutrients. Root exudates, like organic acids, amino acids, phenolics, and sugars, facilitate their uptake by root hairs, leading to concentration in roots. Processes like inactivation, complexation, and precipitation within roots enable the metals to move to aboveground tissues (Souri *et al.*, 2019). Detoxification mechanisms in plants, like antioxidant enzymes and vacuolar sequestration, facilitate the further translocation of metals to aerial parts.

Metal ions stored in vacuoles can enter the xylem stream through root symplasm, and ultimately reach shoots (Pasricha *et al.*, 2021).

Bai *et al.* (2018) explored the bioaccumulation potential of *Potamogeton malaianus*, *Nymphoides peltata*, *Eichhornia crassipes*, and *Hydrilla verticillata*, highlighting their ability to accumulate Cu, Cr, Mn, Ni, Zn, Co, Pb, and V in different plant organs. Studies by Nazir *et al.* (2011) reported the maximum value of Zn accumulation in the shoots of *Xanthium strumarium* and *Brachiaria repens*, while *Phragmites australis* and *Rubus ulmifolius* showed high concentrations of Zn in the root, indicating Zn accumulation primarily in the roots compared to other plant parts (Marques *et al.*, 2007). Khankane and Varshaney (2008) analysed the accumulation of heavy metals by weeds along drains in Jabalpur, reporting that *A. philoxeroides* accumulates higher concentrations of Ni and Zn, followed by *Mollugo verticillata* and *Amaranthus viridis* in their different parts. Malik *et al.* (2010) studied heavy metal contamination and accumulation in soil and wild plant species in the industrial area of Islamabad, concluding a higher accumulation of Cu in shoots followed by Zn, Cr, Pb, Co, and Ni. Murtić *et al.* (2021) assessed the phytoremediation potential of eight native plant species in heavy metal-contaminated soils along the Spreča river valley. Their findings revealed that *Artemisia vulgaris* and *Trifolium repens* had significantly higher concentrations of toxic heavy metals in their shoots, indicating their effectiveness in heavy metal removal.

This study also aimed to assess the potential of *A. tenella* for metal accumulation. Species with high BCFs, BACs, and TFs for a heavy metal are considered to have the potential for remediating that specific metal (Sabo and Laden, 2018). *A. tenella* showed comparatively higher values of these coefficients which shows its potential for phytoremediation of the heavy metal contaminated environment. Fontem Lum *et al.* (2014) evaluated the phytoremediation potential of some plants in the Bassa Industrial Zone of Douala in terms of BACs, TFs, and BCFs. They identified the phytoextraction potentials of *Dissotis rotundifolia* and *Kyllinga erecta* for Pb, *Paspalum orbiculare* for Fe, and the phytostabilization potentials of *Eleusine indica* and *K. erecta*

for Cu- and Pb-contaminated soils. Sabo and Ladan (2018) reported *C. dactylon*, *Cyperus rotundus*, and *Cenchrus biflorus* as potential phytostabilizers due to their high BAC and BCF values. In the present study, *C. dactylon* and *C. rotundus* were dominant in many quadrats. Results from Ogunkunle *et al.* (2014) indicated that *Sida acuta* and *Pennisetum purpureum* have the potential for phytostabilizing Cr, Cd, and Zn-contaminated soils, as well as for phytoextracting Cr and Cd, owing to their high TF value. Heavy metal accumulation and translocation were assessed in *Cannabis sativa*, *Silybum marianum*, and *Rumex dentatus* growing in the industrial area of Rawalpindi (Zehra *et al.*, 2009), and their phytoremediation potential was studied using BCFs. Ahmad *et al.* (2023) investigated heavy metal accumulation in 17 wild plant species from industrial areas and found that most exhibited $TF > 1$, indicating their ability to translocate metals from roots to shoots. Notably, *Solanum xanthocarpum* and *Argemone Mexicana* showed higher concentrations of metals such as Pb and Cr in their aerial parts. Ratheesh-Chandra *et al.* (2018) calculated the BCFs and BTCs of some wild plants growing in metal-contaminated soil, finding that *A. tenella* exhibited significant values exceeding one for Cr, Cu, Fe, Ni, and Pb.

The present study also highlighted the dominance of invasive alien species in riparian areas of industrial regions. Most of the plants in these habitats were herbaceous perennials. The high level of invasion in riverside areas is attributed to significant anthropogenic disturbances. This alarming proportion of alien species poses a serious threat to the region, as it increases the likelihood of invasive plants spreading to distant areas. Additional impacts include the loss of native habitats and species, disruption of water flow, alteration of groundwater regimes, and changes in biomass (Kacheche and Mzuza, 2021). This study not only provides baseline knowledge on species composition and diversity patterns in these critical vegetation zones but also emphasizes the need for effective management strategies to mitigate the spread of invasive plants in the region.

Hence, the phytosociological study on the riparian zone of Walayar Riverbank identified *A. tenella* as the most dominant and consistently distributed species. Its ecological prevalence along this disturbed area suggests a combination of morphological resilience and physiological adaptability especially in an environment exposed to heavy metal influx from industrial activities. Given this prominence, the next chapter examines the morphological, anatomical and physiological responses of *A. tenella* to heavy metal toxicity.

3.1. Introduction

Toxic heavy metal (HM) contamination poses significant stress on plants which adversely affects their growth, development, and physiological functions. Among the HMs, cadmium (Cd), chromium [Cr(VI)] and lead (Pb) are particularly toxic as they disrupt various morphological, anatomical and physiological processes in plants (Srivastava *et al.*, 2021). Understanding the impact of these metals on plant structure and metabolism is crucial for assessing plant responses to metal-induced stress. *Alternanthera tenella* Colla is an invasive weed species that thrives in riparian zones near metal industries and exhibits a potential tolerance to heavy metal exposure. This chapter explores alterations in the structure and metabolic processes induced by these metal stresses in *A. tenella* to provide insights into its adaptive mechanisms and tolerance potential.

3.1.1. Source of heavy metals

The wide distribution of heavy metals such as Cd, Cr(VI) and Pb in soil, water and biological systems has become a significant environmental concern over the past few decades (Gill *et al.*, 2016). These metals are released into the environment from both natural and anthropogenic sources such as atmospheric deposition, industrial discharges, mining and agricultural activities. Mining, copper and nickel smelting, synthetic phosphate fertilizers, electronic waste recycling, fossil fuel combustion, and leachates generated from landfill sites, compost and sludges are the major sources of Cd contamination (Genchi *et al.*, 2020). Chromium ions are introduced into the environment through industrial activities such as chrome plating, cement production, steel manufacturing, dye and paint production, mining, leather tanning, textile processing, and the aircraft industry. Improper disposal of waste in sanitary landfills can lead to Cr leaching into surrounding ecosystems (Haider *et al.*, 2022; Coetzee *et al.*, 2020). Similarly, Pb pollution stems from various sources, including smelting, mining operations, coal combustion, waste incineration, battery recycling, Pb-based paints, leaded aviation gasoline, water pipes, solder, and the production of lead shot and fishing sinkers (Raj and Das, 2023).

3.1.2. Forms and toxicity of metals

Cadmium exists primarily in the forms of Cd^{2+} and Cd-chelates in soil solutions (Abedi and Mojiri, 2020). It is a highly toxic, widespread industrial and environmental pollutant. Cd has been labelled as a Group 1 human carcinogen by IARC (IARC 1993), a Group 2a carcinogen by the Environmental Protection Agency (EPA), and a 1B carcinogen by the European Chemical Agency (IPCS 1992; ATSDR 2012). Chromium possesses a high redox potential and can exist in a range of valence states, from (-II) to (VI), with Cr(0), Cr(III), and Cr(VI) being the most stable forms in nature (Jiang *et al.*, 2015). The different oxidation states of Cr exhibit varying chemical, toxicological, and epidemiological characteristics. Cr(VI) is 100 times more toxic than Cr (III) due to its higher oxidation potential, solubility and mobility (Qianqian *et al.*, 2022; Liang *et al.*, 2021). Due to its high toxicity and cancer-causing properties, Cr(VI) is declared as Group 1 human carcinogen by the International Agency for Research on Cancer (IARC) and as Group A carcinogen by World Health Organization (WHO) (Georgaki *et al.*, 2023). Lead (atomic mass 207.2 u) is a naturally occurring toxic metal found in the earth's crust. It is rarely found free in nature and is usually seen in ores as oxides, sulphides, sulphates, or carbonates. Lead exposure to living systems remains a major public health issue. WHO reported that Pb exposure was responsible for nearly half of the 2 million deaths caused by known chemical exposures in 2019. As a result, WHO identified Pb as one of the ten chemicals of major public health concern requiring urgent global action.

3.1.3. Entry into plants

Plants absorb Cd, Cr and Pb through various pathways depending on their chemical forms and mobility in the environment. Cd enters plant roots in both inorganic and organic complex forms due to its high mobility and assimilability. Cd is efficiently translocated from the roots to the shoots in its ionic form (Kubier *et al.*, 2019; Dong *et al.*, 2019). This mobility and ability to accumulate in aerial plant tissues make Cd a significant contaminant in the food chain. Chromium is absorbed by plants in both its stable valence states i.e., Cr(III) and Cr(VI) (Shahid *et al.*, 2017).

Cr(III) enters plant cells passively through cation exchange sites present in the cell walls (Park, 2020). Cr(VI) is actively transported into plant cells via sulphate and phosphate carriers due to its chemical similarity to these anions (Xu *et al.*, 2021; Gill *et al.*, 2017). Lead enters plants through both soil and atmospheric pathways. While atmospheric Pb can enter through stomatal openings during cellular respiration, free Pb ions are absorbed from the soil via capillary action or calcium (Ca^{2+})-permeable channels (Sharma and Dubey, 2005). Pb ions are largely retained in the roots due to the restriction of radial transport by Casparian strip in the endodermis (Collin *et al.*, 2022; Kumar *et al.*, 2017). This limits the translocation of Pb into shoots and thereby minimize Pb toxicity in above-ground plant tissues.

3.1.4. Effects on plants and humans

Cd, Cr and Pb pose serious risks to plant and human health due to their negative effects on different physiological processes. Visible symptoms of Cd toxicity are chlorosis, stunted growth and plant necrosis. Cd adversely impacts physiological processes such as photosynthesis, seed germination, enzyme activities, stomatal conductance, transpiration and the uptake and transport of essential minerals (Nazar *et al.*, 2012; Zhang *et al.*, 2020). High mobility of Cd allows it to easily enter the human body and replaces calcium in biological systems. Historical cases such as the Itai-Itai disease in Japan highlight the devastating effects of Cd contamination including kidney failure, painful degenerative bone disease and gastrointestinal and lung disorders (Nishijo *et al.*, 2017).

Cr(VI) have detrimental effects on plant development, seed germination, mineral nutrition, photosynthesis, metabolism, biomass production, crop productivity and ultimately leads to plant death (Singh *et al.*, 2020; Sehrish *et al.*, 2019). Cr toxicity reduces plant growth by inducing ultrastructural modifications of cell membranes and chloroplasts, disrupting cell division and the cell cycle, degrading chlorophyll, and causing water and mineral imbalances. It also impairs transpiration, nitrogen assimilation and enzymatic activities (Zaheer *et al.*, 2020; Ugwu and Agunwamba, 2020). Cr exposure can act as a powerful epithelial irritant, leading to bronchitis, dermatitis, and tuberculosis in humans (Giri and Singh, 2017; Saud *et al.*, 2022).

Lead toxicity in plants results in rapid inhibition of root growth, chlorosis, blackened root systems and stunted overall growth (Nas and Ali, 2018). Pb interferes with plant processes such as seed germination, seedling development, photosynthesis, transpiration, mineral uptake and water content. It also disrupts enzymatic activities and induces oxidative stress through excessive production of ROS leading to lipid peroxidation, inhibition of ATP synthesis and DNA damage (Porrut *et al.*, 2011). Pb toxicity has severe consequences for human health. Traditional medicines including Ayurvedic preparations have been found to contain Pb that implies its widespread presence. Pb in fine particulate matter can penetrate deep into the lungs of humans and animals and approximately 40% of inhaled Pb entering the bloodstream (Lin *et al.*, 2020). Once absorbed, Pb is distributed and accumulated in vital organs such as the brain, liver, kidneys and bones. Pb exposure is responsible for 30% of idiopathic intellectual disabilities, 3% of chronic kidney diseases and 4.6% of cardiovascular diseases globally (WHO, 2023). There is no safe level of Pb exposure due to its severe toxicity which makes its elimination from the environment a global priority.

The accumulation of Cd, Cr and Pb in the food chain and their adverse effects on plant and human health emphasize the urgent need for efficient strategies such as phytoremediation to mitigate these heavy metals in the environment.

3.1.5. Plants reported for phytoremediation of heavy metals

The efficiency of phytoremediation mostly depends on the ability of plants to accumulate heavy metals and the total biomass production. Therefore, the plants used for phytoremediation should be able to accumulate heavy metals in their tissues and grow on metalliferous soils without showing toxic effects. Hyperaccumulator plants play a crucial role in phytoremediation by primarily absorbing HMs from the soil. Previous reports pointed out that several species could be used, such as *Celosia argentea*, *Chromolaena odorata*, *Conyza sumatrensis*, *Gynura pseudochina*, *Nicotiana tabacum*, *Salix mucronata*, *Cassia alata*, *Solanum melonaena*, and *Kummerowia striata* for Cd phytoremediation (Raza *et al.*, 2020) and *Cynodon dactylon*, *Jatropha curcas*, *Leersia hexandra*, *Pluchea indica*, *Phragmites australis*,

Typha angustifolia, *Pteris vittata*, *Nopalea cochenillifera* and *Pennisetum sinense* for Cr phytoremediation (Chen *et al.*, 2020). No plants have been reported to naturally phytoextract Pb, but species like *Sesbania drummondii*, *Brassica juncea*, *Zea mays*, *Pelargonium hortorum*, and *Sedum alfredii* are known for chelator-assisted phytoextraction of Pb (Kafle *et al.*, 2022). However, the effectiveness of most of these hyperaccumulator plants in phytoremediation is limited due to their slow growth and low biomass, making them less practical for large-scale applications.

3.1.6. Plant selection and scope of study

Selecting plant species for phytoremediation requires an understanding of the morpho-physiological changes that occur in plants under stress. However, *ex vitro* studies are challenging since environmental factors significantly influence anatomical and physiological responses. Unlike that, *in vitro* cultivation techniques provide an effective approach for heavy metal studies as they isolate the effects of individual stressors under controlled investigations (Rodrigues *et al.*, 2017; Muszyńska *et al.*, 2018; Martins *et al.*, 2020). Several species within the Amaranthaceae family are recognized for their ability to accumulate heavy metals in their biomass which makes them promising candidates for phytoremediation (Rodrigues *et al.*, 2017; Kanwal *et al.*, 2019; Odiyi *et al.*, 2019). The genus *Alternanthera* is well known for remediating contaminated environments owing to their competitive growth and high stress tolerance (Yan *et al.*, 2023; Kanwal *et al.*, 2021; Mazumdar and Das, 2015). Among this genus, *A. tenella* has shown high resilience in industrially polluted areas.

Alternanthera tenella Colla (syn. *Alternanthera ficoidea* (L.) Sm.) is a flowering plant in the Amaranthaceae family native to the Caribbean and South America. It is widely introduced across tropical regions and is considered invasive in many countries. The species propagates through seeds and grows as a prostrate herb with white-villous stems. Its leaves measure 3-4.5 x 2 cm are elliptic-oblong with a sub-acute apex, and exhibit a hairy upper surface. The plant features axillary, ovoid, sessile flower heads with spinescent bracts and tepals. The reproductive structures include

five fertile stamens, oblong anthers, and strap-shaped pseudo-staminodes, while its seeds are brownish and discoid (<https://indiabiodiversity.org>).



Fig. 3.1. Whole plant of *Alternanthera tenella*, showing its general growth habit and morphological features.

A. tenella has been used for its diuretic, antipyretic, antiseptic, anti-inflammatory and anti-diarrheal properties in traditional medicine (Biella Cde *et al.*, 2008). The secondary metabolites in plant include tannins, saponins, phytols, carotenoids and polyphenols along with bioactive compounds such as vitexin, quercetin, kaempferol, amarantin, betaine and isoamarantin (Deladino *et al.*, 2017). Its pharmacological potential including antibacterial, immunomodulatory, anti-tumor and hypolipidemic effects has been studied (Pandey *et al.*, 2019).

A. tenella is a facultative metallophyte and an invasive alien species in India that exhibits tolerance to various metal stresses (Chinmayee *et al.*, 2014). Critical gaps remain despite the progress in understanding invasive alien plant species (IAPS) in phytoremediation. Previous studies on *A. tenella* have focused on its ability to accumulate metals such as Cd and Cu under controlled conditions (Clairvil *et al.*, 2022; Rossini *et al.*, 2022; Martins *et al.*, 2020; Rodrigues *et al.*, 2017). However, its mechanisms for tolerating and accumulating non-essential toxic metals are poorly

understood. This study aims to bridge this knowledge gap by thoroughly examining the morphological, structural and physiological responses of *A. tenella* to heavy metal stress, specifically Cd, Cr and Pb.

A. tenella offer unique advantages due to its invasive nature and robust growth under extreme conditions which reduces the need for introducing non-native species for remediation. A deeper understanding of the specific mechanisms by which *A. tenella* tolerates and accumulates heavy metals could improve its potential use in environmental management and remediation of highly contaminated areas. Hence, the study was conducted to analyse the morphological changes and structural modifications in *A. tenella* tissues under selected heavy metal (Cd, Cr, Pb) toxicity, to assess the physiological responses including photosynthetic efficiency and stress biomarkers to HM toxicity and to evaluate HM uptake and translocation within different tissues of *A. tenella*.

3.2. Review of literature

Heavy metals are major inorganic contaminants with high density and atomic weight. Heavy metal contamination due to industrial effluents and sewage disposal poses serious risks since they do not biologically degrade. These metals harm plants and affect consumers as they move up the food chain (Ghori *et al.*, 2019).

3.2.1. Impact of heavy metal pollution on plant systems

Heavy metal pollution deteriorates soil quality and disrupts plant physiological and molecular processes (Angon *et al.*, 2024; Hafeez *et al.*, 2023). Toxic metals cause morphological abnormalities and metabolic disorders that lead to lower crop yields (Amari *et al.*, 2017). In plants, heavy metal stress interferes with essential enzymatic functions and protein structures by replacing vital metal ions in biomolecules and damaging cell membranes (Hasan *et al.*, 2017). These disruptions affect photosynthesis, respiration, and homeostasis resulting in impaired photosynthetic pigments, photosystems, and stomatal conductance (Souri *et al.*, 2019). High metal concentrations directly inhibit cytoplasmic enzymes and trigger oxidative stress producing ROS that damages cell structure and plant metabolism (Mansoor *et al.*,

2023). Heavy metals interact with the functional groups of biomolecules such as O-H, C-H, C-C, C-O and C=O and thereby modify polysaccharides, lipids and proteins (Peng *et al.*, 2020; Usman *et al.*, 2019).

They also have indirect toxic effects such as displacing essential nutrients from cation exchange sites which further harms plant health (Chukwu and Gulser, 2025). Long-term exposure causes chlorosis, necrosis, turgor loss, poor seed germination, and senescence (Angulo-Bejarano *et al.*, 2021). Heavy metals also disrupt soil microbial activity and reduce enzyme activity important for plant metabolism (Campillo-Cora *et al.*, 2025). This interference leads to growth inhibition and ultimately, plant death. Managing heavy metal toxicity in agricultural systems is essential for maintaining soil health, sustaining plant productivity, and ensuring food safety.

3.2.2. Cadmium

Cd contamination mainly comes from anthropogenic activities such as metal industries, transportation, landfills, mining, fuel combustion, sewage sludge, synthetic phosphate fertilizers and urban waste disposal (Haider *et al.*, 2021; Bigalke *et al.*, 2017). These activities increase Cd levels in soils and water bodies, allowing it to enter plants and creating serious environmental and health concerns (Kubier *et al.*, 2019).

Cd uptake and transport in plants depend on species, soil properties and environmental conditions. Cd enters roots through apoplastic and symplastic pathways and competes with essential nutrients such as Ca, Mg and Zn. After absorption, Cd moves through the xylem to the shoots and leaves and can also translocate via phloem to fruits and seeds although most Cd remains localized in roots. Soil pH, acidification and root exudates increase Cd solubility and uptake. Membrane transporters also regulate the absorption and translocation of Cd across cell membranes and tissues (Haider *et al.*, 2021; Shaari *et al.*, 2022).

Cd is easily absorbed by plants and severely affects their growth, development and metabolism (Ahmad *et al.*, 2019). Toxic symptoms usually appear when Cd levels in

plant tissues exceed 3 to 30 mg kg⁻¹ (Ismael *et al.*, 2019). Cd reduces growth and biomass by disturbing water balance and nutrient transport (Ullah *et al.*, 2020). Cd toxicity caused growth inhibition, cell death and membrane damage in Soybean (Holubek *et al.*, 2020). Cd also disrupts membrane integrity by inducing oxidative stress, altering lipid composition and inhibiting ATPase activity that further impairs cellular homeostasis and transport functions (Shaari *et al.*, 2022).

Cd exposure alters root morphology in *Arabidopsis* by reducing the elongation zone and meristematic tissue while increasing root hair density (Li *et al.*, 2023). Cd accumulation causes gradual thickening of root and leaf tissues in *A. tenella* (Rodrigues *et al.*, 2017). Root diameter increases due to expansion of cortical tissue and parenchymal cells which improves resistance to solute flow (Ismael *et al.*, 2018). Non-hyperaccumulators develop border-like cells at the root tip to inhibit Cd entry and this feature is absent in hyperaccumulators. Apoplastic barriers and deposition of lignin and suberin further restrict Cd mobility by reducing cell wall penetrability and immobilizing Cd in the apoplast (Li *et al.*, 2023).

Cd toxicity reduces root water content and primary root elongation. This causes root dieback, suppresses secondary root growth, and thickens cell walls. It also decreases aquaporin activity and restricts water uptake through both apoplast and symplast pathways (Haider *et al.*, 2021). Cd accumulation increases osmotic pressure causing plants to produce proline and ABA to manage water deficit stress (Zulfiqar *et al.*, 2022). Cd also reduced leaf area, chlorophyll, carotenoids, stomatal density and cell size, and it suppresses hypocotyl elongation and chloroplast development (Haider *et al.*, 2021; Rasafi *et al.*, 2022). These changes lower crop yield, and decrease protein, starch and sugar content in grains, while increasing Cd concentration in grain and proline levels in leaves (Ismael *et al.*, 2019; Huybrechts *et al.*, 2019). Plants exclude Cd from guard and subsidiary cells to protect their stomata (Sohail *et al.*, 2019). Cd replaces essential ions such as Mg²⁺ in chlorophyll and Ca²⁺ in the Ca/Mn clusters of the PSII complex damaging the photosynthetic machinery (Rasafi *et al.*, 2022). It interferes with chlorophyll biosynthesis, the Calvin

cycle and respiration while inducing lipid peroxidation, and reducing photosynthetic efficiency and total plant productivity (Zulfiqar *et al.*, 2022).

Cd also induces stomatal closure and inhibits Fe reductase activity causing Fe deficiency and reduced photosynthesis (Haider *et al.*, 2021). PSII and the oxygen-evolving complex (OEC) are major targets of Cd toxicity. In tobacco, OEC was protected by Trx CDSP32 under Cd stress (Li *et al.*, 2023). Cd disrupts electron transport and suppresses PSII photoactivation (Farooq *et al.*, 2016). These effects reduce nitrogen fixation, nitrogen assimilation and protein and carbohydrate synthesis which leads to plant growth retardation (Zulfiqar *et al.*, 2022).

Cd induces oxidative stress primarily by disrupting the antioxidant defence system rather than by directly producing free radicals (Li *et al.*, 2023; Ghori *et al.*, 2019). This results in lipid peroxidation, ROS accumulation, reduced NADPH levels and inhibition of photo-assimilation enzymes. Free radicals damage biomolecules by blocking functional groups and displacing essential ions such as Ca^{2+} (Rasafi *et al.*, 2022). To counteract oxidative stress, plants activate antioxidant defences including enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and glutathione reductase (GR) and non-enzymatic antioxidants like ascorbate (AsA), glutathione (GSH), carotenoids, alkaloids, tocopherols, proline, and phenolic compounds (flavonoids, tannins, and lignin) (Sharma *et al.*, 2012; Michalak, 2006; Rastgoo *et al.*, 2011).

SOD activity increases consistently under Cd stress and converts O_2^- into H_2O_2 (Haider *et al.*, 2021; Li *et al.*, 2023). Its increase has been observed in soybean, tobacco, and cotton under Cd exposure (Guo *et al.*, 2019; Khan *et al.*, 2013; Buzduga *et al.*, 2022), though in some cases Cd reduces SOD activity. CAT detoxifies H_2O_2 in peroxisomes (Gomes *et al.*, 2017). Under Cd exposure, CAT responses vary by species and Cd concentration. *Groenlandia densa* and *Crotalaria juncea* maintains higher CAT activity, while *Canna orchoides* and cucumber showed reduced CAT activity (Munawar *et al.*, 2022; Zhang *et al.*, 2021; Yilmaz *et al.*, 2011; Pereira *et al.*, 2002). APX localized in chloroplasts and cytoplasm and the heme protein GPX detoxify H_2O_2 by converting it to water. Both APX and GPX activity shows

increased activity under Cd exposure in species like wheat and *Ceratophyllum demersum*, where both GPX and APX activity increase in response to Cd toxicity (Haider *et al.*, 2021; Gomes *et al.*, 2017; Tran and Popova, 2013). Cd also influences non-enzymatic defences. In some studies, Cd increased phenolics and MDA without significantly affecting flavonoids, vitamin C, or proline content (Sani *et al.*, 2017).

Cd stress increases expression of heat shock proteins (HSPs), proteinases, and pathogenesis-related proteins, but suppresses proteins involved in primary metabolic pathways such as glycolysis, the Calvin cycle, and the Krebs cycle (Zulfiqar *et al.*, 2022; Haider *et al.*, 2021). Cd disrupts protein function by altering their structure and replacing cofactors such as Zn^{2+} and Ca^{2+} , while its affinity for $-\text{OH}$ and $-\text{COOH}$ groups in DNA leads to chromosomal alterations and impaired gene expression (Rasafi *et al.*, 2022).

3.2.3. Chromium

Chromium uptake and accumulation in plants vary according to plant type and Cr speciation. Cr^{6+} is absorbed actively via sulphate and phosphate transporters, while Cr^{3+} enters through ion channels by simple diffusion/osmosis (Gomes *et al.*, 2017). Root-to-shoot transport occurs via the xylem, and Cr^{6+} is reduced to Cr^{3+} at the endodermis and further in aerial parts (Guo *et al.*, 2021). Soil factors such as pH, redox-active substances and redox potential influence the reduction of Cr^{6+} to Cr^{3+} which affects Cr bioavailability and toxicity (Srivastava *et al.*, 2021). Cr forms complexes with organic ligands in the rhizosphere and ferric reductase helps the conversion of Cr^{6+} to Cr^{3+} . Cr^{3+} binds strongly to cell walls and restricts its mobility (Srivastava *et al.*, 2021).

Chromium toxicity inhibits seed germination, root and shoot growth, biomass and yield (Ali *et al.*, 2023; Ao *et al.*, 2022). Cr stress also disrupts water potential, transpiration, nutrient uptake, photosynthesis and chlorophyll biosynthesis (Gomes *et al.*, 2017). Although low Cr levels may promote growth in few species, overall, its effects are harmful (Saud *et al.*, 2022; Srivastava *et al.*, 2021).

Cr exposure decreases root elongation, diameter, surface area and formation of and secondary roots and root hairs. Cr induces structural changes such as expanded pith and cortex, root cap degradation, reduced lateral root formation and diminished mitotic activity with anomalous cell division (Ao *et al.*, 2022; Gomes *et al.*, 2017). Stem growth and biomass also decline due to disrupted nutrient and water transport and altered internal structure (Ao *et al.*, 2022; Srivastava *et al.*, 2021).

Studies on *Hibiscus esculentus* shows the phytotoxic impacts of Cr including reduced seed germination, seedling vigor, chlorophyll content and tolerance indices (Amin *et al.*, 2013). Leaves under Cr stress shows reduced area, lower chlorophyll a/b and carotenoid contents, chlorosis, necrosis, wilting, plasmolysis in root cells, premature aging, and leaf shedding (Gomes *et al.*, 2017; Srivastava *et al.*, 2021; Ao *et al.*, 2022). Stomatal changes include reduced wax deposition and wider openings. *Pteris* showed an increase in palisade and spongy mesophyll cell numbers under Cr stress (Srivastava *et al.*, 2021). Cr toxicity reduces chlorophyll a, chlorophyll b, and carotenoid levels, with chlorophyll b more affected than chlorophyll a. Cr also induces pheophytinization of chlorophyll molecules (Ao *et al.*, 2022).

Cr impairs photosynthesis by disrupting electron transport in photosystems I and II, binding to cytochrome heme groups, and replacing Mg^{2+} in RuBisCO. This interference inhibits carbon assimilation, photophosphorylation and RuBisCO activity (Gomes *et al.*, 2017; Zulfiqar *et al.*, 2023). Damage to PSII is pronounced due to LHCII sensitivity. Mitochondrial electron transport chain is also inhibited under Cr stress (Srivastava *et al.*, 2021). Chloroplast function is further disrupted by thylakoid disorganization and inhibition of Calvin cycle enzymes (Ao *et al.*, 2022). Cr also reduces stomatal conductance and evapotranspiration (Gomes *et al.*, 2017).

Cr stress decreases water potential, transpiration rate, relative water content, and longitudinal water movement. It induces stomatal closure, increasing osmotic pressure and limiting leaf water capacity (Srivastava *et al.*, 2021; Zulfiqar *et al.*, 2023). Root damage caused by Cr stress restricts water absorption and

apoplastic/symplastic transport, although tissue-level adaptations may enhance water retention to aid survival (Zulfiqar *et al.*, 2023).

Cr stress disrupts nutrient homeostasis by reducing uptake of essential macro- and micronutrients (P, Fe, Cu, Mg, Zn, Ca, S) through adsorption site blockage and formation of insoluble complexes. Some species show increased accumulation of P, Mn, and Zn, while S and Cu normally decline (Ao *et al.*, 2022; Zulfiqar *et al.*, 2023). In rice, Mg accumulated in mitochondria and nucleus under Cr stress (Srivastava *et al.*, 2021). Damage to plasmodesmata and inhibition of plasma membrane H⁺-ATPase also impair mineral uptake efficiency (Zulfiqar *et al.*, 2023).

Cr toxicity generates excess ROS, causing oxidative stress that disrupts cellular homeostasis with effects including DNA damage, protein fragmentation, lipid peroxidation, pigment degradation, and cell death (Ao *et al.*, 2022; Gomes *et al.*, 2017). Unstable Cr intermediates (Cr⁴⁺ and Cr⁵⁺) increase ROS production and impair plant growth by inhibiting cell division and accelerating cell damage (Zulfiqar *et al.*, 2023). Plants counter this stress by activating antioxidant enzymes such as CAT, GPX, APX, POD, SOD, GR, DHAR and GST. Increased activity of these enzymes under Cr stress has been reported in plants like *Vigna radiata*, *Brassica juncea* and tomato (Elkelish *et al.*, 2024; Sharma *et al.*, 2022; Ahammed *et al.*, 2025). Proline levels also rise under Cr³⁺ exposure (Ali *et al.*, 2023; Zulfiqar *et al.*, 2023). Cr stress increases sucrose content in rice seedlings by enhancing the activities of sucrose phosphate synthetase (SPS), sucrose synthase (SS) and soluble acid invertase (AI) showing modulation of carbohydrate metabolism (Guo *et al.*, 2021; Srivastava *et al.*, 2021). Cr stress reduces concentration of phenolic compounds (Zulfiqar *et al.*, 2023). Enhanced ROS levels correlate with increased MDA content under Cr stress (Zulfiqar *et al.*, 2023).

3.2.4. Lead

Major sources of Pb pollution include ore and metal processing, automotive emissions, mining, burning, leaded gasoline, industrial smelting and combustion,

paint, batteries, water pipes, pottery, boat building, the arms and glass industries, fertilizer and pesticide use, pigments, and book printing (Ankush *et al.*, 2023).

Pb uptake in plants occurs mainly via apoplastic pathways and Ca^{2+} -permeable channels and is influenced by soil conditions, root surface area, and microbial associations (Zulfiqar *et al.*, 2019; Kumar *et al.*, 2020). Pb accumulates predominantly in roots, followed by leaves, stems, inflorescences, and seeds, with limited translocation to aerial parts due to barriers including the Casparian strip in the endodermis and exodermis (Collin *et al.*, 2022; Kohli *et al.*, 2019). Pb retention mechanisms include vacuolar sequestration, cell wall binding, precipitation as insoluble salts, and accumulation on the plasmalemma (Kumar *et al.*, 2017; Collin *et al.*, 2022; Kohli *et al.*, 2019; Aslam *et al.*, 2021).

Pb toxicity disrupts seed germination, root elongation, seedling growth and overall plant development. It impairs chlorophyll biosynthesis, transpiration, cell division, membrane stability and enzyme activity. Excessive ROS generation causes ATP inhibition, lipid peroxidation, and DNA damage which affects water balance and protein stability (Kumar *et al.*, 2017). Visible symptoms of Pb toxicity include root blackening, chlorosis, biomass loss, necrotic lesions and reduced water uptake (Giannakoula *et al.*, 2021; Bejarano *et al.*, 2021). Pb also reduces flower production and sucrose content disrupting carbohydrate synthesis (Collin *et al.*, 2022).

Pb competes with Mn^{2+} , Fe^{2+} , Mg^{2+} , Ca^{2+} and K^{+} reducing nutrient uptake and translocation. K^{+} deficiency under Pb toxicity is common due to their similar atomic radii and shared transport pathways (Zulfiqar *et al.*, 2019). Pb also inhibits nitrogen metabolism by suppressing nitrate reductase and restricting phosphorus uptake (Kohli *et al.*, 2019). Pb exposure affects vascular development causing thinner leaf blades, impaired xylem and phloem differentiation and narrower xylem vessels in Soybean (Nas and Ali, 2018). Pb impairs photosynthesis and reduces total chlorophyll, chlorophyll a and b and carotenoid levels. It accelerates chlorophyll degradation and suppresses enzymes involved in pigment synthesis such as ferredoxin NADP⁺ reductase and ALAD involved in chlorophyll synthesis (Nas and Ali, 2018; Zulfiqar *et al.*, 2019). Pb damages chloroplast structure, limits PSII

electron transport, alters thylakoid membrane composition, reduces grana stacking and inhibits Calvin cycle enzymes. This results in reduced CO₂ fixation and photosynthetic efficiency (Nas and Ali, 2018; Zulfiqar *et al.*, 2019; Kohli *et al.*, 2019). Pb stress lowers turgor, alters water regulation, causes stomatal closure and reduces transpiration and water use efficiency (Pinho and Ladeiro, 2012; Ruciska-Sobkowiak *et al.*, 2013). Plants tolerate Pb through selective uptake, excretion, ligand binding, and vacuolar sequestration (Kumar *et al.*, 2020; Collin *et al.*, 2022). The Casparian strip and root endodermis serve as physical barriers that restrict radial Pb movement.

Plants also activate enzymatic and non-enzymatic antioxidants such as proline, glutathione, APX, GPX, CAT, SOD and GR to reduce harmful effects of ROS (Thakur *et al.*, 2017). Studies on *Vallisneria natans* revealed that Pb first increases antioxidant activity followed by declines in CAT, SOD and ascorbic acid after prolonged exposure (Wang, 2012). In *Tetraena qataranse*, Pb causes a sustained increase in APX, GPX, GR, SOD and CAT showing strong tolerance and hyperaccumulation potential (Usman *et al.*, 2019). Similar observations were noted in wheat, rice and *Cassia angustifolia* (Aslam *et al.*, 2021; Verma and Dubey, 2003). Phenols can directly chelate Pb ions. Increased phenylpropanoids, flavanols and anthocyanins confirm their antioxidant role. Total phenolic content in wheat rise under Pb stress but declines at high Pb levels, indicating complex polyphenol involvement in ROS generation during metal stress (Kohli *et al.*, 2019). Pb exposure increases MDA and electrolyte leakage that are indicative of increased lipid peroxidation and oxidative stress (Zulfiqar *et al.*, 2019; Kohli *et al.*, 2019; Aslam *et al.*, 2021).

3.2.5. Plants for heavy metal remediation

Heavy metal contamination including Cd, Cr and Pb poses serious environmental and health risks, making effective remediation essential. Phytoremediation, microbial action and chemical amendments help extract, stabilize and sequester metals (Zulfiqar *et al.*, 2022). Phytoremediation is a cost-effective and eco-friendly technology that use plants to remove or stabilize heavy metals and improve soil

health (Berti and Cunningham, 2000; Ali *et al.*, 2013). Advanced detoxification methods include the use of hyperaccumulator plants, antioxidant mechanisms, phytostabilization and phytoextraction (Srivastava *et al.*, 2021).

Plant-based and microbial strategies show potential for metal removal. Hyperaccumulators convert Cd into less toxic forms, while nutrients and organic amendments improve soil conditions and reduce Cd uptake (Luo and Zhang, 2021). Plant growth-promoting bacteria (PGPB) increase Cr resistance by modifying root architecture and secreting metal-sequestering molecules (Sharma *et al.*, 2020). Organic and inorganic amendments also help stabilize Cr-contaminated soils (Ao *et al.*, 2022). Other advanced methods include chemical priming, nanopriming, biotechnological approaches, microbe-assisted remediation and genetic breeding of metal-safe cultivars (Ali *et al.*, 2023; Zulfiqar *et al.*, 2023; Aslam *et al.*, 2021).

Over 400 plant species including *Arabidopsis*, *Thlaspi*, *Brassica*, and *Sedum alfredii* show strong phytoremediation ability (Lone *et al.*, 2008). Aquatic species such as *Eichhornia crassipes*, *Echinochloa stagnina*, and *Phragmites australis* effectively accumulate Cd, Ni and Pb for phytoextraction and phytostabilization (Eid *et al.*, 2020). Additional hyperaccumulator species such as *Trifolium repens*, *Helianthus petiolaris*, *Zea mays* and *A. thaliana* also remove metals efficiently (Makarova *et al.*, 2021; Saran *et al.*, 2020; Awa and Hadibarata, 2020; Sakakibara *et al.*, 2010). Some plants show high phytoremediation efficiency including *Pennisetum sinense* for Cr (Chen *et al.*, 2020), *Tetraena qataranse* for Cd, Cr, Cu and Ni (Usman *et al.*, 2019), *Chenopodium alba* and *Solanum viarum* for remediation of mining sites (Mohan *et al.*, 2019; Afonso *et al.*, 2019). Research also highlights *Eclipta alba* and *A. philoxeroides* as effective phytoremediators (Sharma *et al.*, 2021). Genetic modification and breeding have improved tolerance and metal accumulation capacity in crops such as rice (Arif *et al.*, 2019; Khan *et al.*, 2015). Morphological and phytochemical changes under metal stress also help optimize phytoremediation for sustainable environmental restoration (Fattahi *et al.*, 2021).

3.2.6. Studies on metal stress in *Alternanthera* Species

Members of the Amaranthaceae family are well known for heavy metal accumulation and are widely used in phytoremediation (Chinmayee *et al.*, 2012;

Abba and Ibrahim, 2017; Odiyi *et al.*, 2019). *Suaeda fruticosa* stabilizes trace metals in its roots and relies on antioxidant activity for stress tolerance (Bankaji *et al.*, 2016). *Chenopodium quinoa* responds differently to Cd and Pb where Pb remains safe for consumption and Cd accumulates in grains (Amjad *et al.*, 2022). *Amaranthus retroflexus* also shows stress tolerance and detoxification potential (Alsherif *et al.*, 2022). *Aerva lanata* exhibits physiological and biochemical variations between polluted and non-polluted environments (Neema and Jisha, 2020).

Anoliefo *et al.* (2008) identified *Alternanthera* species as strong candidates for phytoremediation. Species such as *A. tenella*, *A. sessilis*, *A. dentata* and *A. philoxeroides* are common hyperaccumulators (Wijeyaratne *et al.*, 2021; Gajbhiye and Bhalerao, 2017; Jha *et al.*, 2016; Tiwari *et al.*, 2021). *A. bettzickiana* also shows phytoremediation ability under Cd, Cu and Pb stress due to its physiological and biochemical responses (Khalid *et al.*, 2020; Kanwal *et al.*, 2019; Tauqeer *et al.*, 2016). *A. sessilis* can accumulate Cr and Pb in roots and leaves and can hyperaccumulate Cd. *A. sessilis* (Wijeyaratna and Kumari, 2021). It also accumulates high concentrations of Fe, Cu, Cr, Zn and Ni (Gajbhiye and Bhalerao, 2017). *A. dentata* showed better metal tolerance and accumulates Cd, Cr, As and Ni (Tiwari *et al.*, 2021). *A. philoxeroides* accumulates Cu, Pb, Zn etc. and can be used as phytoremediation agent (Hong-Bo *et al.*, 2010; Lin *et al.*, 2007). It efficiently uptakes and translocates Pb, Cd and Cr under field conditions (Jha *et al.*, 2016). *A. ficoidea* shows high arsenic accumulation, and uptakes Pb, Cd, and Cr effectively (Jha *et al.*, 2016; Vajravel and Saravanan, 2014).

A. sessilis and *A. tenella* show increased antioxidant activity and tolerance under Cd and Pb stress (Chinmayee *et al.*, 2013; Chinmayee *et al.*, 2014). *In vitro* studies showed that *A. tenella* can accumulate Cd and tolerate up to 100 μM Cd by adjusting its anatomy and physiology (Rodrigues *et al.*, 2017). Its morphophysiological responses, bioaccumulation ability and Cu tolerance have also been documented under *in vitro* conditions (Martins *et al.*, 2020; Cuchiara *et al.*, 2013). Cu treatments impair PSI electron transport and alter mineral nutrients and photosynthetic pigments in *A. tenella* (Cuchiara *et al.*, 2013). Cd causes

photochemical dysfunction in *A. tenella*, whereas Zn addition ameliorates Cd-induced stress by improving the photosynthetic pigments and plant performance (Clairvil *et al.*, 2021; Clairvil *et al.*, 2022). Co-treatment with Si reduces Cd-induced physiological damage in *A. tenella*, resulting in enhanced growth and Cd tolerance (Rossini *et al.*, 2022).

Although some studies have looked at the phytoremediation potential of *A. tenella* and explored the individual aspects of its response to heavy metal stress, a comprehensive understanding remains unlimited. Most previous research has focused on isolated physiological or biochemical parameters often in *in vitro* conditions. Integrative studies simultaneously examining morphological, anatomical, and physiological adaptations of *A. tenella* to the comparative effects of non-essential toxic heavy metals (Cd, Cr, Pb) were clearly lacking. The mechanistic links between the structural changes and the related physiological responses such as nutrient allocation, antioxidant enzyme activity and metal sequestration strategies also remain unclear. With the increasing environmental levels of these metals and the importance of *A. tenella* as a potential hyperaccumulator, this study addresses these gaps. The study improves our understanding of metal tolerance and phytoremediation potential of *A. tenella* through multi-level analyses of its responses to these all three metals.

3.3. Materials and methods

3.3.1. Plant material

Alternanthera tenella Colla, an invasive plant species from the family Amaranthaceae, was selected for the present study. Healthy *A. tenella* stem cuttings that had a consistent size (25–30 cm) were selected for the experiment. The cuttings were kept in distilled water for a week to induce rooting. Later, the rooted plantlets were transferred to ½ strength Hoagland's solution (50 mL) taken in glass test tubes (25 × 150 mm) for 21 days. The plantlets were maintained in a controlled environment with a relative humidity of $60 \pm 5\%$ and a temperature of $30 \pm 2^\circ\text{C}$ to optimize plant growth throughout the study. The rooted plantlets were later

subjected to standardized concentrations of heavy metal salts for a duration of 21 days using hydroponic culture techniques.

3.3.2. Chemicals and instruments

Either AR or GR grade chemicals were purchased from MERCK (Sigma-Aldrich) SRL, and HIMEDIA companies. UV-VIS spectrophotometer (UV-1900i, Shimadzu Corporation, Kyoto, Japan), visible spectrophotometer (ELICO SL 177), and refrigerating centrifuge (Eppendorf 5804 R) were used for the analysis.

3.3.3. Treatment with heavy metals

$K_2Cr_2O_7$, $Cd(NO_3)_2$, and $Pb(CH_3COO)_2$ were selected respectively for inducing Cr, Cd, and Pb stress. Plants were separately subjected to 21-day treatments with various concentrations of $K_2Cr_2O_7$, $Cd(NO_3)_2$, and $Pb(CH_3COO)_2$ ranging from 0 to 700 μM , prepared from a 1M stock solution, with five replicates for each concentration. The range of concentrations was gradually narrowed down, initially with a difference of 50 μM and later with a difference of 10 μM between concentrations. Following screening, the concentrations of metal salts that induced substantial stress in rooted propagules of *A. tenella* but exhibited approximately 50% growth inhibition were selected as standardised treatment levels. So, for further study, healthy plantlets of *A. tenella* were exposed to these standardised concentrations of HMs for 21 d under hydroponic culture conditions provided with $\frac{1}{2}$ strength Hoagland medium (Hoagland No. 2 Basal salt mixture, HiMedia). Plants grown in medium without metals were used as a control.

3.3.4. Sampling

A. tenella samples were collected on 0 d, 7 d, 14 d, and 21 d after metal treatment. 5 biological replicates were taken for each treatment. The morphological and anatomical parameters were measured. The roots, stems, and leaves of the sampled plants were separated after weighing and were immediately frozen in liquid nitrogen to store for physio-chemical analysis. Hereupon, $K_2Cr_2O_7$, $Cd(NO_3)_2$,

$\text{Pb}(\text{CH}_3\text{COO})_2$ and treatments will be referred to as chromium stress (Cr), cadmium stress (Cd), and lead stress (Pb) respectively.

3.3.5. Plant growth

The whole plants were harvested at specific intervals of HM exposure, washed with distilled water and blotted dry. The growth of *A. tenella* under different HMs was evaluated by measuring root and shoot length, leaf area, dry weight, and tolerance index.

3.3.5.1. Root and shoot length, leaf area

The roots, stems and leaves were washed with distilled water prior to measurement and then blotted dry with filter paper. The length of roots and shoots were measured with a graduated scale. The outline of the newly opened leaves was sketched on a graph paper and the leaf area was calculated.

3.3.5.2. Tolerance index percentage (TI)

Tolerance index percentage of the plantlets was calculated according to the equation given by Turner (1994).

$$\text{TI} = \frac{\text{Observed value of root length in solution with metal} \times 100}{\text{Observed value of root length in solution without metal}}$$

3.3.6. Anatomical modifications

The fresh roots, stems and leaves of *A. tenella* subjected to both control and metal treatments were harvested after 21d. After sectioning, these samples were immersed in 2.5% glutaraldehyde solution in a 0.1 M phosphate buffer (pH 7.2) at room temperature for overnight fixation. Following fixation, the specimens were rinsed with distilled water and dehydrated using a series of increasing ethanol concentrations (20%, 40%, 60%, 70%, 80%, 90%, and 99%), with each step lasting 10 minutes. The dehydrated samples were dried and mounted on aluminium stubs using double-sided carbon tape. After gold sputtering, the specimens were viewed using a Jeol 6390LA scanning electron microscope (SEM) operating at 20 kV to

capture digital micrographs (Soumya *et al.*, 2022). Cell dimensions like thickness and diameter of xylem were measured using Image - J (1.48G version) software.

3.3.7. Element analysis in tissues

The dehydrated root, stem, and leaf samples of *A. tenella* were examined under a high-resolution scanning electron microscope (SEM, Jeol 6390LA) at a magnification of $\times 300,000$ for analysing their elemental distribution patterns. A quantitative compositional analysis of the elements was also performed using an Inca analyzer EDX spectrophotometer and the method described by Cocozza *et al.* (2008). Using EDXMA (energy dispersive X-ray microanalysis), three microspots in the root, stem, and leaves from both control and metal-treated plantlets were analysed. The three microspots were named spectrum 1, 2, and 3 respectively.

3.3.8. Physiological and biochemical analysis

3.3.8.1. Biomass determination

For the estimation of biomass, the plants were carefully removed from the nutrient solution, washed thoroughly in water, and blotted to dryness. Fresh weight of plants was determined using weighing balance. Then these were oven dried initially at 100°C for one h and then at 60°C till the weight become constant. Total biomass per sample was calculated from fresh weight and dry weight in grams.

3.3.8.2. Leaf Relative Water Content (RWC)

Relative water content was determined following the method described by Barrs (1968) and Boyer (1968). Freshly collected leaves were weighed and then immersed in water overnight to achieve full turgidity. The leaves were blot dried and weighed next day to obtain their turgid weight. The leaves were then dried in an oven at 70°C, and their dry weight was recorded. The RWC (%) was calculated using the following formula:

$$\% \text{ RWC} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100$$

Where, FW=Fresh Weight, DW=Dry weight, TW=Turgid Weight.

3.3.8.3. Estimation of photosynthetic efficiency of the leaf

Estimation of photosynthetic pigments (chlorophyll, carotenoids) were done according to Arnon (1949). Fresh leaves of control and treated plants were washed with water and blotted using filter paper. To estimate chlorophyll and carotenoids, 80% acetone was used as the extracting medium.

One hundred milligram fresh leaf samples, both control and treated, were homogenized using mortar and pestle in 20 ml of 80% aqueous acetone (1:10 w/v) at room temperature, followed by centrifugation at 5000 rpm for 5 min. The supernatant was collected, and the residue was re-extracted with 80% acetone and centrifuged. This process was repeated until the residue became colourless. The final volume of the pooled supernatant was recorded, and its absorbance was measured at 470 nm, 663 nm, and 645 nm using spectrophotometer against the solvent blank (80% acetone). The concentrations of chlorophyll a (chl-a), chlorophyll b (chl-b), total chlorophyll (chl-t), and carotenoids were determined as mg chlorophyll and carotenoids per gram fresh weight using the following formula.

$$\text{Chlorophyll a} = 12.7 (A_{663}) - 2.69 (A_{645}) V/1000 \times W$$

$$\text{Chlorophyll b} = 22.9 (A_{645}) - 4.68 (A_{663}) V/1000 \times W$$

$$\text{Total Chlorophyll} = 20.2 (A_{645}) + 8.02 (A_{663}) V/1000 \times W$$

$$\text{Carotenoids} = 1000 (A_{470}) + 3.27 (\text{chl. a} - \text{chl. b}) V/229 \times W$$

Where, A_{663} - Absorbance at 663 nm, A_{645} - Absorbance at 645 nm, A_{470} - Absorbance at 470 nm, V - Volume of extract, W - Weight of tissue.

3.3.8.4. Estimation of total proteins

The total protein content in the tissues (root, stem, and leaf) of *A. tenella* was determined according to the method described by Lowry *et al.* (1951), using Folin-Ciocalteu reagent. Bovine serum albumin (BSA, 66 KDa) was used as the standard protein.

Extraction

Two hundred milligram of each sample (200 mg) was homogenized in in 5 ml of phosphate buffer (pH 7) using pre-chilled glass mortar and pestle. An equal volume of 10% trichloroacetic acid (TCA) was added to a known volume of homogenate pipetted in a centrifuge tube. The mixture was refrigerated for 1 h for flocculation. After centrifuging for 10 minutes, the supernatant was decanted off and the residue was washed with 2% trichloroacetic acid (w/v), followed by washing each with diethyl ether, 30% perchloric acid (v/v) and 80% acetone (v/v) to remove lipids, starch and pigments respectively. The resulting pellet was extracted with 5 ml of 0.1N NaOH, boiled for 5 min in water bath, cooled, and then centrifuged. The resulting supernatant was then transferred to test tubes and used for protein estimation.

Estimation**Reagents**

- A - 2% Sodium carbonate in 0.1N sodium hydroxide
- B - 0.5% Copper sulphate in 1% potassium sodium tartarate
- C - Alkaline copper sulphate solution: Prepared by mixing 50 ml
A and 1 ml of B reagents prior to use.
- D - Folin-Ciocalteu reagent

Suitable aliquots were taken in duplicates from each extract. Volume was made up to 1 ml with double distilled water. The 5 ml of reagent C was added to each tube, mixed well, and allowed to stand for 10 min at room temperature. To this mixture, 0.5 ml of 1N Folin-Ciocalteu reagent was added, mixed, and the tubes were incubated for 30 min in dark at room temperature to facilitate colour development. The absorbance of the solution was measured at 700 nm using spectrophotometer and compared to a standard curve prepared with Bovine serum albumin to quantify the protein content in the samples.

3.3.8.5. Estimation of soluble sugars

Total soluble sugars were quantified using the phenol-sulphuric method as described by Dubois *et al.* (1956).

Extraction

Five hundred milligram each of root, stem, and leaf tissues were homogenized in 10 ml of 80% ethanol, followed by centrifugation at 2000 rpm for 20 min. The resulting supernatant was carefully collected.

Estimation

One millilitre of the extract was combined with 1 ml of 5% phenol and 5 ml of concentrated sulphuric acid. The reaction mixture was incubated in a water bath at 26-30°C for 20 minutes. The absorbance of the resulting colour was measured spectrophotometrically at a wavelength of 490 nm. The concentration of soluble sugars in the samples was determined by comparison to a glucose standard curve and expressed as mg g⁻¹ of FW.

3.3.8.6. Estimation of Proline

The free proline content in *A. tenella* tissues was determined using the ninhydrin method described by Bates *et al.* (1973).

Extraction

One-gram fresh tissue of plant parts (root, stem, and leaf samples) was homogenized in 10 ml of 3% (w/v) aqueous sulfosalicylic acid using a clean glass mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 10 min and the supernatant was collected and estimation of proline was done using acid ninhydrin.

Estimation

Two millilitres from the supernatant were taken in test tubes in triplicate and equal volume of glacial acetic acid and acid ninhydrin reagent were added to it.

Following incubation of the reaction mixture for 1 h in a boiling water bath and subsequent cooling on an ice bath, the colour reaction was developed. To extract the chromophore formed during the reaction, 4 ml of toluene was added to the reaction mixture and stirred well for 30 seconds. Then the chromophore-toluene layer was separated carefully, and the colour intensity of the solution was measured at 520 nm using spectrophotometer and proline concentration in the samples was determined by comparison to a standard curve prepared using L-proline.

3.3.8.7. Estimation of total phenolics

Total phenolics were estimated using the method described by Bray and Thorpe (1954). Gallic acid was used as the standard.

Extraction

One hundred milligram each of plant tissue was homogenized in 2.5 ml of 75% methanol (v/v) in a clean mortar and pestle. The homogenate was centrifuged at 2000 rpm for 20 min and the supernatant was collected.

Estimation

Aliquots of 0.1 ml of supernatant in triplicate were pipetted out. 0.5 ml of Folin-Ciocalteu reagent and 8.9 ml of distilled water were added to it. The mixture was left at room temperature for 3 minutes. 0.5 ml of 25% sodium carbonate was added, and the reaction was allowed to proceed for 1 h for colour development. The absorbance of the developed colour was spectrophotometrically at a wavelength of 725 nm using spectrophotometer. The concentration of phenols present in the samples was expressed as Gallic acid equivalent (GAE) in g kg⁻¹ of sample.

3.3.8.8. Estimation of malondialdehyde (MDA) content

The MDA content estimation was done according to Li *et al.* (2010).

Extraction

Five hundred milligrams each of root, stem, and leaf was homogenised in 1.5 mL of 5% TCA. The homogenate was centrifuged at 5000 rpm for 10 min. The supernatant was collected and used for the estimation of MDA.

Estimation

Two millilitres of supernatant (in triplicates) was mixed with an equal aliquot of 0.67% thiobarbaturic acid (TBA). The solution was heated for 30 min at 95-100°C, cooled, and then centrifuged at 5000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm, 600 nm, and 450 nm against the reagent blank using spectrophotometer. The absorbance value at 532 nm was corrected for non-specific turbidity by subtracting absorbance value at 600 nm; and then the MDA content was calculated as: $6.45(A_{532} - A_{600}) - 0.56 A_{450}$.

3.3.8.9. Estimation of Flavonoids

Total flavonoids were measured by the colorimetric aluminium chloride method described by Mervat *et al.* (2009). Quercetin was used as standard.

Extraction

Five hundred milligrams of the plant tissues was macerated in 50 ml of 80% ethanol. After 1 day of incubation at room temperature, the extract was centrifuged for 15 min at 10,000 rpm. The supernatant was collected and used for the estimation of flavonoids.

Estimation

Aliquots were taken in triplicate from each extract and the volume was made up to 1 ml with methanol. 0.1 ml $AlCl_3 \cdot H_2O$ (10%), 0.1ml of potassium acetate and 2.8 ml distilled water were added, mixed and incubated at room temperature for 30 min. The absorbance of the solution was measured at 415 nm using a spectrophotometer. The concentrations of flavonoid in the test samples were calculated from the standard calibration plot and expressed as mg quercetin equivalent /g of sample.

3.3.8.10. Catalase (CAT) assay

Catalase activity was determined in fresh plant material according to Aebi (1984).

Five hundred milligrams each of the leaf, stem and root samples was homogenized in a chilled glass mortar and pestle in phosphate buffer (50 mM, pH 7.0) and 2 g

polyvinylpolypyrrolidone (PVPP, 1% w/v). The extract was made up to 10 ml using phosphate buffer and then centrifuged for 15 min at 16000 rpm in refrigerated centrifuge at 4°C. The supernatant was used for the enzyme assay.

Assay system (3 mL total volume) consisted of 1 mL of 50 mM potassium phosphate buffer (pH 7.0) and 2 mL of enzyme extract in a quartz cuvette. The reaction was initiated by adding 1 mL of 30 mM H_2O_2 to the reaction mixture. The absorbance decrease ($\Delta\text{Abs}/\text{min}$) was read at $\lambda = 240 \text{ nm}$ for 30 min at 5 min intervals with the UV-VIS spectrophotometer.

3.3.8.11. Ascorbate peroxidase (APX) assay

Ascorbate peroxidase activity was measured according to Chen and Asada (1989).

Five hundred milligrams of fresh sample (leaf, stem and root) was homogenized in a pre-chilled mortar and pestle in 50 mM Tris HCl buffer (pH 7.5) and 50 mg PVPP. The filtered homogenate was made up to 10 ml and centrifuged at 16000 rpm for 15 min at 4°C in refrigerated centrifuge. The supernatant was used for the enzyme assay.

Ascorbate peroxidase (APX) activity was determined by monitoring the decline of absorbance at 290 nm as ascorbate was oxidized. The reaction mixture (3 mL) contained 100 mM potassium phosphate buffer (pH 7.5), 0.2 mM H_2O_2 , 0.5 mM sodium ascorbate, and the enzyme extract. Absorbance ($\Delta\text{Abs}/\text{min}$) was read at $\lambda = 290 \text{ nm}$ after 30 s for 3 min with the UV-VIS spectrophotometer.

3.3.8.12. Guaiacol peroxidase (GPX) assay

Guaiacol Peroxidase (GPX) Activity was assessed using the method described by Velikova *et al.* (2000).

Five hundred milligrams of plant tissues was homogenized in 50 mM Tris HCl buffer (pH 7.5) and 50 mg PVPP in pre-chilled mortar and pestle. The homogenate was filtered and made up to 10 ml using phosphate buffer. The mixture was then

centrifuged at 16000 rpm for 15 min in refrigerated centrifuge (Eppendorf model) at 4°C. The supernatant was used for enzyme assay.

Assay system contained 2.7 mL of 50 mM phosphate buffer (pH 7.8), 1% (v/v) guaiacol, and 100 µL of the enzyme extract. The reaction was initiated by adding 12 µL H₂O₂ (0.3%) to the reaction mixture and absorbance ($\Delta\text{Abs}/\text{min}$) was read at $\lambda = 470\text{nm}$ after 30 s for 3 min with the UV-VIS spectrophotometer.

3.3.8.13. Superoxide dismutase (SOD) assay

Superoxide dismutase activity was determined by inhibiting the photochemical reduction of nitroblue tetrazolium (NBT), as outlined by Kumar *et al.* (2012).

Five hundred milligrams each of plant tissue were homogenized using a chilled glass mortar and pestle in phosphate buffer (50 mM, pH 7.8) and 100 mg PVPP and was made up 10 mL using phosphate buffer. The homogenate was then centrifuged at 16000 rpm for 15 min at 4°C in refrigerated centrifuge. The supernatant was used for enzyme assay.

The assay mixture consisted of phosphate buffer (50 mM, pH 7.6), EDTA (0.1 mM), sodium carbonate (50 mM), L-methionine (12 mM), NBT (50 µM), riboflavin (10 µM), and enzyme extract (100 µL) in a final volume of 3.0 mL. A control reaction without the crude extract was also performed. The SOD reaction was carried out by exposing the reaction mixture to white light for 15 min at room temperature. Absorbance was then measured at 560 nm using a UV-VIS spectrophotometer. One unit (U) of SOD activity was defined as the enzyme activity causing 50% inhibition of photochemical reduction of NBT.

3.3.9. Fourier transform infrared (FTIR) analysis

The dried powder of leaf, stem, and root samples was mixed with dried, water-free KBr at a ratio of 1:150 mg (sample: KBr). The mixture was then subjected to a hydraulic pressure of 10 tons to form KBr discs suitable for infrared (IR) analysis. These discs were positioned in the path of the instrument beam of a Fourier-transform infrared (FT-IR) spectrometer (JASCO 4100, Shanghai, China) to measure

the solid-state spectrum. The IR analysis was conducted within the range of 400 - 4000 cm^{-1} with a resolution of 2 cm^{-1} . Spectral data were compared against reference spectra to identify the functional groups present in the samples (Kumar *et al.*, 2015).

3.3.10. Biolocalization of heavy metals

The freshly collected samples on 21 d for total heavy metal content determination (root, stem, and leaves) were dried at 100°C for 1 h and then placed at 60°C until a constant dry weight was achieved. The dried samples were ground and finely powdered. About 0.2 g of dried plant samples were weighed and left in a mixed solution of nitric and hydrochloric acids (4HNO₃: 1HCl) in closed PTFE vessels, followed by digestion in a microwave oven for two hours at 200°C. The concentrations of metals in the *A. tenella* digest were measured using an inductively coupled plasma-atomic emission spectrometer (ICP-AES) (Perkin Elmer Avio 200, USA) (Hansen *et al.*, 2013). The metal accumulation and rate in plant and distribution proportion of metals in roots were calculated using formulas given by Fang *et al.* (2017). Biological accumulation coefficient (BAC), translocation factor (TF) and bioconcentration factor (BCF) were calculated to assess the phytoremediation potential of *A. tenella* against each heavy metal stress using the formulas of Yanqun *et al.* (2005), Yoon *et al.* (2006) and Zayed *et al.* (1998).

$$\text{TF} = [\text{metal}]_{\text{shoot}} / [\text{metal}]_{\text{root}}$$

$$\text{BAC} = [\text{metal}]_{\text{shoot}} / [\text{metal}]_{\text{solution}}$$

$$\text{BCF} = [\text{metal}]_{\text{plant tissue}} / [\text{metal}]_{\text{solution}}$$

3.3.11. Statistical analysis

Data is presented as mean $\pm$ standard error (SE) obtained from three independent experiments with each experiment comprising five replicates ($n = 15$). Different lowercase letters within a row indicate significant differences among treatments at the same day ($p < 0.05$). Different uppercase letters within a column indicate significant differences among days within the same treatment ($p < 0.05$). Normality of the data was assessed using the Shapiro-Wilk test ($p < 0.05$). Analysis of variance

(ANOVA) was conducted, followed by Dunnett test, to identify significant differences between the control and metal-treated plants, at $p < 0.05$. Analyses were carried out using SPSS® (version 26.0, IBM Corp, Armonk, NY, USA), and Microsoft Excel® (Professional Plus 2016, Redmond, WA, USA).

3.4. RESULTS

Heavy metal toxicity affected the biochemical and physiological responses in *A. tenella*, causing structural modifications and inhibiting plant growth and development. Treatment of rooted propagules of *A. tenella* with increasing concentrations of Cd, Cr, and Pb resulted in progressive growth inhibition and visible stress responses. Among the tested ranges, concentrations that caused approximately 50% inhibition of growth were identified as standardized levels for subsequent experiments.

Exposure to different concentrations of Cd revealed an exact dose-dependent inhibition of plant growth in *A. tenella* (Table 3.1). Control plants exhibited a steady increase in root length, stem length, and leaf area throughout the experimental period. At lower concentrations (50–100 μM), treated plants remained viable and showed moderate reduction in growth compared to control. At 150–170 μM , a significant inhibition of root and shoot elongation and a marked reduction in leaf area were observed, corresponding to nearly % growth inhibition relative to control. At 180 μM and above of Cd exposure, plant growth was severely impaired, and plants failed to survive beyond 21 days. Based on these observations, 170 μM Cd was selected as the standardized concentration for subsequent experiments.

Table 3.1. Effect of different concentrations of Cd concentration on *A. tenella* at equal intervals, for a period of 21 days.

Cd conc. (μM)	Days	Root length (cm)	Shoot length (cm)	Leaf area (cm^2)
0	0	6.4 $\pm$ 0.28	39.94 $\pm$ 0.52	1.82 $\pm$ 0.13
	7	8.4 $\pm$ 0.28	42.66 $\pm$ 0.53	2.7 $\pm$ 0.21
	14	10.8 $\pm$ 0.21	49.38 $\pm$ 0.44	3.06 $\pm$ 0.16
	21	13.26 $\pm$ 0.32	53.42 $\pm$ 0.38	3.7 $\pm$ 0.2
50	0	6.46 $\pm$ 2.1	39.96 $\pm$ 0.2	1.79 $\pm$ 0.12

	7	9.02±2.1	40.2±0.15	2.55±0.09
	14	9.81±1.3	41.2±0.7	2.56±0.07
	21	10.08±1.9	43.87±0.23	2.88±0.12
100	0	6.46±2.1	39.96±0.2	1.77±0.03
	7	7.86±2.2	42.76±0.18	1.98±0.03
	14	9.14±3.1	43.13±0.45	2.14±0.12
	21	9.88±2.3	43.93±0.12	2.23±0.11
150	0	6.46±2.1	39.96±0.2	1.78±0.1
	7	8.72±2.4	41.46±0.25	1.88±0.12
	14	8.72±2.4	41.9±0.12	1.96±0.05
	21	9.06±1.1	42.6±0.13	1.99±0.06
160	0	6.46±2.1	39.96±0.2	1.83±0.05
	7	7.98±1.5	40.83±0.23	1.86±0.12
	14	8.15±1.8	41.76±0.31	1.96±0.02
	21	8.23±2.3	41.88±0.33	1.96±0.02
170	0	6.4±0.28	39.94±0.52	1.78±0.08
	7	7.44±0.15	40.78±0.26	1.9±0.07
	14	7.62±0.17	41.44±0.37	1.92±0.07
	21	7.78±0.24	41.48±0.38	1.92±0.07
180	0	6.46±2.1	39.96±0.2	1.81±0.01
	7	7.08±3	40.83±0.45	1.83±0.1
	14	7.56±2.8	40.91±0.13	1.83±0.1
	21	Plant died		
190	0	6.46±2.1	39.96±0.2	1.83±0.01
	7	7.14±1.6	40.01±0.12	1.68±0.01
	14	Plant died		
	21	Plant died		
200	0	6.46±2.1	39.96±0.2	1.8±0.02
	7	7.45±1.3	39.97±0.3	1.41±0.01
	14	Plant died		
	21	Plant died		

Cr exposure with increasing concentrations also had pronounced effect on the growth of *A. tenella* in a dose-dependent manner (Table 3.2). Plants in control showed consistent increases in root and shoot elongation and leaf area over 21

days. Plants maintained moderate growth inhibition at lower concentrations (50–150 μM), with evident slight reductions in shoot length and leaf area compared to control. Significant inhibition of plant growth was observed under concentrations 200–240 μM , with root elongation and leaf expansion reduced to nearly half of control values. At concentrations ≥ 250 μM , plant growth was severely restricted, and plants failed to survive beyond 14 days. Based on these observations, 240 μM was identified as the standardized concentration that produced substantial stress with approximately 50% growth inhibition and was selected for further experiments.

Table 3.2. Effect of different concentrations of Cr concentration on *A. tenella* at equal intervals, for a period of 21 days.

Cr conc. (μM)	Days	Root length (cm)	Shoot length (cm)	Leaf area (cm^2)
0	0	6.4 $\pm$ 0.28	39.94 $\pm$ 0.52	1.82 $\pm$ 0.13
	7	8.4 $\pm$ 0.28	42.66 $\pm$ 0.53	2.7 $\pm$ 0.21
	14	10.8 $\pm$ 0.21	49.38 $\pm$ 0.44	3.06 $\pm$ 0.16
	21	13.26 $\pm$ 0.32	53.42 $\pm$ 0.38	3.7 $\pm$ 0.2
50	0	6.46 $\pm$ 2.1	39.96 $\pm$ 0.2	1.79 $\pm$ 0.12
	7	9.02 $\pm$ 2.1	40.2 $\pm$ 0.15	2.55 $\pm$ 0.09
	14	9.81 $\pm$ 1.3	41.2 $\pm$ 0.7	2.56 $\pm$ 0.07
	21	10.08 $\pm$ 1.9	43.87 $\pm$ 0.23	2.88 $\pm$ 0.12
100	0	6.46 $\pm$ 2.1	39.96 $\pm$ 0.2	1.77 $\pm$ 0.03
	7	7.86 $\pm$ 2.2	42.76 $\pm$ 0.18	1.98 $\pm$ 0.03
	14	9.14 $\pm$ 3.1	43.13 $\pm$ 0.45	2.14 $\pm$ 0.12
	21	9.88 $\pm$ 2.3	43.93 $\pm$ 0.12	2.23 $\pm$ 0.11
150	0	6.46 $\pm$ 2.1	39.96 $\pm$ 0.2	1.78 $\pm$ 0.1
	7	8.72 $\pm$ 2.4	41.46 $\pm$ 0.25	1.88 $\pm$ 0.12
	14	8.72 $\pm$ 2.4	41.9 $\pm$ 0.12	1.96 $\pm$ 0.05
	21	9.06 $\pm$ 1.1	42.6 $\pm$ 0.13	1.99 $\pm$ 0.06
200	0	6.46 $\pm$ 2.1	39.96 $\pm$ 0.2	1.83 $\pm$ 0.05
	7	7.98 $\pm$ 1.5	40.83 $\pm$ 0.23	1.86 $\pm$ 0.12
	14	8.15 $\pm$ 1.8	41.76 $\pm$ 0.31	1.96 $\pm$ 0.02
	21	8.23 $\pm$ 2.3	41.88 $\pm$ 0.33	1.96 $\pm$ 0.02
210	0	6.46 $\pm$ 2.1	39.96 $\pm$ 0.2	1.78 $\pm$ 0.08

	7	7.44±0.15	40.78±0.26	1.9±0.07
	14	7.62±0.17	41.44±0.37	1.92±0.07
	21	7.78±0.24	41.48±0.38	1.92±0.07
220	0	6.46±2.1	39.96±0.2	1.81±0.1
	7	7.44±2.4	40.14±0.8	1.84±0.1
	14	7.59±1	40.98±1.1	1.88±0.08
	21	7.66±2.2	41.1±0.8	1.89±0.02
230	0	6.46±2.1	39.96±0.2	1.82±0.05
	7	7.48±1.5	40.83±0.23	1.84±0.12
	14	7.6±2.2	41.76±0.31	1.86±0.2
	21	7.62±1.4	41.88±0.33	1.86±0.2
240	0	6.46±2.1	39.96±0.2	1.81±0.01
	7	7.08±3	40.83±0.45	1.83±0.1
	14	7.56±2.8	40.9±0.13	1.83±0.2
	21	7.56±2.8	40.91±0.13	1.83±0.2
250	0	6.46±2.1	39.96±0.2	1.83±0.01
	7	7.14±1.6	40.01±0.12	1.86±0.01
	14	Plant died		
	21	Plant died		
260	0	6.46±2.1	39.96±0.2	1.8±0.02
	7	7.45±1.3	39.97±0.3	1.81±0.01
	14	Plant died		
	21	Plant died		

The response of *A. tenella* to Pb exposure was also a concentration-dependent pattern (Table 3.3), Control plants exhibited a steady increase in root length, shoot length, and leaf area over 21 days. At lower concentrations (200–400 μM), growth was only slightly reduced, and plants remained healthy. Growth inhibition became more pronounced at 600–660 μM , as indicated by the reduced shoot elongation and leaf expansion. Plants showed clear signs of stress and substantial reduction in growth at 670–680 μM . At ≥ 690 μM , lethality occurred, and plants failed to survive beyond 14–21 days. From these findings, 680 μM Pb was identified as the standardized concentration for further experiments.

Table 3.3. Effect of different concentrations of Pb concentration on *A. tenella* at equal intervals, for a period of 21 days.

Pb conc. (μM)	Days	Root length (cm)	Shoot length (cm)	Leaf area (cm^2)
0	0	6.46 $\pm$ 2.1	39.96 $\pm$ 2.3	1.83 $\pm$ 0.2
	7	8.56 $\pm$ 3.2	42.86 $\pm$ 2.41	2.74 $\pm$ 0.3
	14	10.74 $\pm$ 2.3	49.22 $\pm$ 3.54	3.12 $\pm$ 0.4
	21	13.29 $\pm$ 2.2	53.31 $\pm$ 2.5	4.45 $\pm$ 0.1
200	0	6.46 $\pm$ 2.1	39.96 $\pm$ 2.3	1.79 $\pm$ 0.12
	7	7.21 $\pm$ 3.1	42.17 $\pm$ 2.15	2.55 $\pm$ 0.09
	14	11.31 $\pm$ 1.3	48.93 $\pm$ 3.7	3.09 $\pm$ 0.7
	21	12.47 $\pm$ 1.9	51.77 $\pm$ 3.2	3.35 $\pm$ 0.12
400	0	6.46 $\pm$ 2.1	39.96 $\pm$ 2.3	1.77 $\pm$ 0.03
	7	8.63 $\pm$ 2.7	43.47 $\pm$ 4.18	2.61 $\pm$ 0.3
	14	9.88 $\pm$ 3.1	45.13 $\pm$ 3.45	2.99 $\pm$ 0.12
	21	11.1 $\pm$ 2.3	49.7 $\pm$ 3.12	3.19 $\pm$ 0.21
600	0	6.46 $\pm$ 2.1	39.96 $\pm$ 2.3	1.78 $\pm$ 0.1
	7	7.32 $\pm$ 3.7	42.17 $\pm$ 2.25	2.64 $\pm$ 0.12
	14	8.77 $\pm$ 2.4	47.77 $\pm$ 1.12	2.84 $\pm$ 0.5
	21	11.32 $\pm$ 1.8	49.2 $\pm$ 2.13	3.19 $\pm$ 0.6
650	0	6.46 $\pm$ 2.1	39.96 $\pm$ 2.3	1.83 $\pm$ 0.05
	7	8.96 $\pm$ 2.5	41.73 $\pm$ 4.23	2.64 $\pm$ 0.12
	14	9.71 $\pm$ 2.8	43.37 $\pm$ 2.31	2.85 $\pm$ 0.02
	21	10.01 $\pm$ 2.3	46.73 $\pm$ 1.33	3.02 $\pm$ 0.2
660	0	6.46 $\pm$ 2.1	39.96 $\pm$ 2.3	1.79 $\pm$ 0.1
	7	7.28 $\pm$ 1.6	40.87 $\pm$ 1.16	1.99 $\pm$ 0.12
	14	8.59 $\pm$ 2.7	42 $\pm$ 1.15	2.1 $\pm$ 0.3
	21	10.57 $\pm$ 3.1	44.5 $\pm$ 2.22	2.78 $\pm$ 0.4
670	0	6.46 $\pm$ 2.1	39.96 $\pm$ 2.3	1.81 $\pm$ 0.1
	7	7.19 $\pm$ 1.6	40.5 $\pm$ 1.16	1.91 $\pm$ 0.12
	14	8.09 $\pm$ 2.7	41.5 $\pm$ 2.15	2.47 $\pm$ 0.3
	21	8.4 $\pm$ 2.9	43.81 $\pm$ 1.22	2.88 $\pm$ 0.4
680	0	6.46 $\pm$ 2.1	39.96 $\pm$ 2.3	1.83 $\pm$ 0.1
	7	7.12 $\pm$ 1.8	40.6 $\pm$ 2.16	1.94 $\pm$ 0.02
	14	7.58 $\pm$ 2.7	41.1 $\pm$ 1.15	2.02 $\pm$ 0.03
	21	8.23 $\pm$ 1.9	41.78 $\pm$ 1.22	2.23 $\pm$ 0.4

690	0	6.46±2.1	39.96±2.3	1.8±0.11
	7	7.12±1.5	40.6±1.45	1.9±0.1
	14	7.14±0.8	37.17±1.13	1.92±0.41
	21	Plant died		
700	0	6.46±2.1	39.96±2.3	1.8±0.2
	7	6.8±1.3	40.1±1.3	1.9±0.1
	14	Plant died		
	21	Plant died		

Preliminary screening with varying concentrations of Cd, Cr, and Pb revealed a clear dose-dependent reduction in growth parameters of *A. tenella*. Control plants showed consistent increases in root and stem elongation and leaf expansion over the 21-day period, while exposure to increasing concentrations of metals resulted in progressive growth inhibition and visible stress symptoms. Lethal effects were observed at higher concentrations of metal. Based on these findings, 170 µM Cd, 240 µM Cr, and 680 µM Pb were selected as the standardised concentrations as they induced substantial stress with approximately 50% growth inhibition, without causing immediate lethality (Table 3.4). These standardized concentrations were used for subsequent experiments. Representative plants treated with the standardized concentrations of Cd, Cr, and Pb for 21 days along with control are shown in Figure 3.2, illustrating the differential growth inhibition and stress responses.

Table 3.4. Concentrations of heavy metal salts used for treatments of *A. tenella* plantlets grown in Hoagland nutrient solution

Name of metal	Name and formula of metal salt	Molecular mass of the salt (g/mol)	Concentration (µM)
Cadmium (Cd)	Cadmium nitrate (Cd(NO ₃) ₂)	236.42	170 µM
Chromium (Cr)	Potassium dichromate (K ₂ Cr ₂ O ₇)	294.185	240 µM
Lead (Pb)	Lead acetate (Pb(CH ₃ COO) ₂ ·3H ₂ O)	379.33	680 µM



Fig. 3.2. Growth performance comparison of *A. tenella* plants of standardized concentrations of Cd, Cr and Pb treatments with control on day 21.

3.4.1. Plant growth

When compared to the control plantlets, all the parameters directly related to plant growth were significantly lowered by the metal treatments throughout the stress period in *A. tenella*.

The root length of *A. tenella* showed a significant reduction under metal treatments. The length of control roots increased from 6.4 ± 0.28 cm to 8.4 ± 0.28 cm on 7d, marking an increase of 31.25%. Cadmium (Cd) and lead (Pb) treatments lead to moderate root growth, with 16.25% and 15.63% increase respectively. Chromium (Cr) had a notable inhibitory effect with only a marginal increase (6.55 ± 0.87 cm) of 2.34% on 7d of treatment, indicating an early growth suppression. By 14d, root growth in control samples further increased by 68.75% from the initial value (10.8 ± 0.21 cm). Cd- and Pb- treated roots reached 8.62 ± 0.17 cm and 9.3 ± 0.28 cm reflecting a 34.69% and 45.31% increase, while Cr-stressed roots showed a mere 3.28% increase over the initial value (6.61 ± 0.14 cm). This trend continued to 21d, when the control root length further increased to 13.26 ± 0.32 cm showing an overall twofold increase during the treatment period. Cr treatment continued to inhibit root elongation with only 6.25% increase (6.8 ± 0.25 cm) over the 21-day period, while Cd- and Pb- treated plants showed a total increase of 37.19% (8.78 ± 0.24 cm) and 72.19% (11.02 ± 0.41 cm) respectively on 21d. Pb stress allowed relatively better root elongation, while Cd had a moderate inhibition on root growth. Cr stress had the most inhibitory effect on root growth throughout the treatment period, with 22%, 38.8%, and 39.74% reduction in root length on 7d, 14d, and 21d compared to the control (Fig. 3.3).

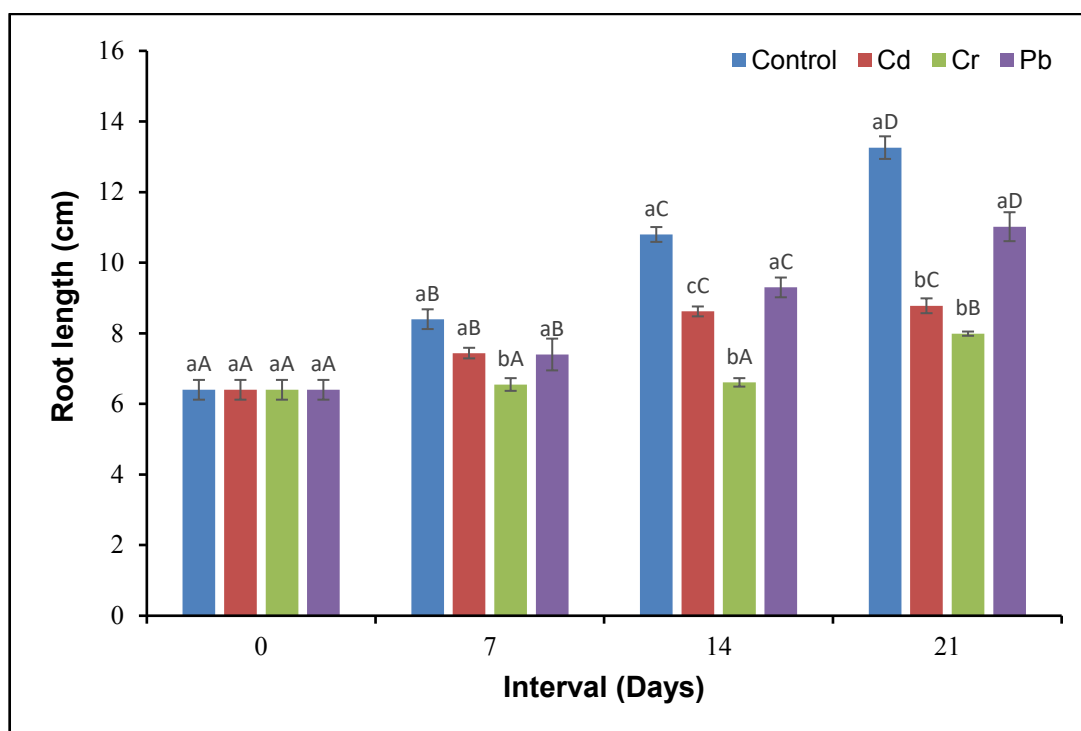


Fig. 3.3. Effect of cadmium, chromium and lead treatments on the root length of *A. tenella*. Data represent the mean $\pm$ SE of five replicates.

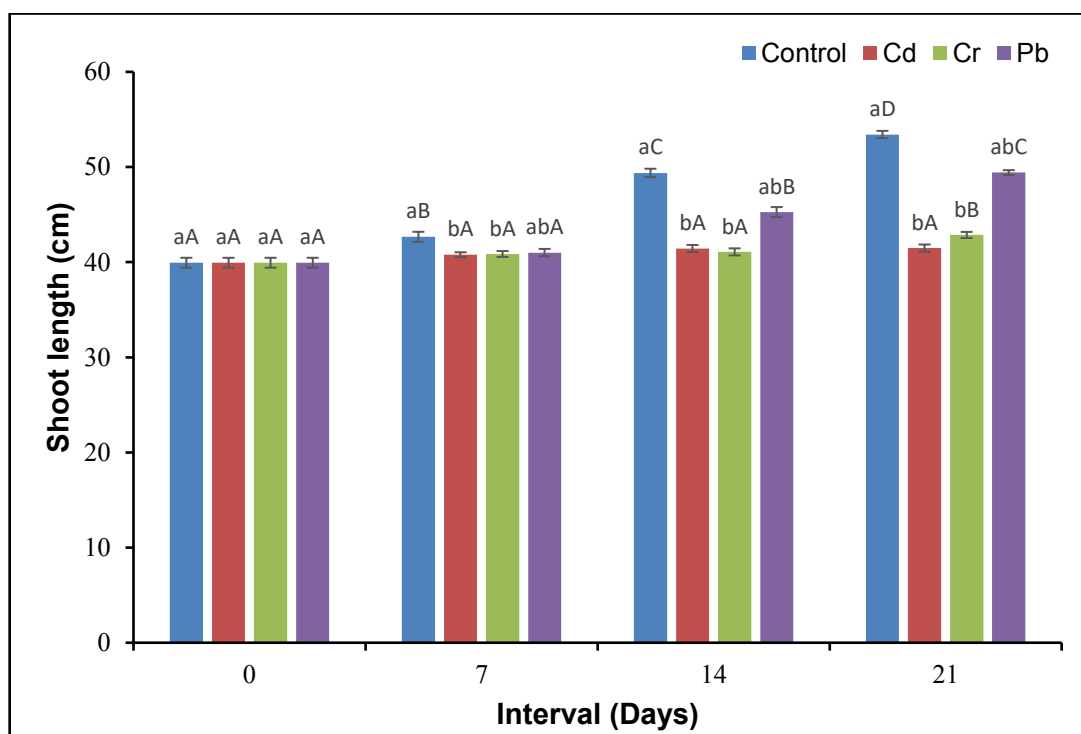


Fig 3.4. Effect of cadmium, chromium and lead treatments on the shoot length of *A. tenella*. Data represent the mean $\pm$ SE of five replicates.

A similar trend was observed in shoot length of *A. tenella*, which decreased under metal treatments. At 0d, the stem length was identical across all treatments (39.94 ± 0.52 cm). Control plants exhibited a slight increase of nearly 6.8% on 7d (42.66 ± 0.53 cm), while all the metal-treated plants showed only marginal of ~2% increase in their shoot growth. By 14d, control plants continued to show substantial elongation with 23.6% increase, reaching 49.38 ± 0.44 cm. Cd- and Cr-treated plants showed very limited shoot growth with 3.8% (41.44 ± 0.37 cm) and 2.9% (41.09 ± 0.31 cm) respectively, while Pb-treated plants had a 13.3% increase in shoot elongation (45.26 ± 0.53 cm) indicating a better tolerance than Cd and Cr. By 21d, the control plants reached the highest stem length (53.42 ± 0.38 cm). Cd-stressed plants remained stagnant (41.48 ± 0.38 cm), while the Cr-treated stems (42.86 ± 0.58 cm) showed slightly better elongation than that of Cd. Pb stress had very minimal impact on shoot elongation, reaching 49.42 ± 0.25 cm (23.7% increase) close to control shoot length. Overall reductions in shoot length of Cd-, Cr-, and Pb-treated plants were 22.3%, 19.8% and 7.5% respectively compared to the control plants (Fig. 3.4).

The area of the newly opened leaves was measured after metal treatments. Both Cd and Cr treatments had high impact on the growth of newly emerged leaves in *A. tenella*. Control leaves exhibited a significant increase in area throughout the stress period. At 0d, leaf area was nearly uniform across all treatments ranging from 1.73 ± 0.07 cm² to 1.86 ± 0.09 cm², with the control at 1.73 ± 0.07 cm². By 7d, control plants showed a 48.35% increase to 2.7 ± 0.21 cm², while Cr and Pb treatments also supported moderate increases to 2.35 ± 0.11 cm² and 2.64 ± 0.12 cm² respectively marking 35.84% and 41.4% rise from their initial values. Cd treatment led to minimal increase in leaf area to 1.9 ± 0.07 cm² (6.7%). By 14d, a 68.13% increase in the leaf area in control plants was observed (3.06 ± 0.16 cm²). Cr- and Pb-treated plants also showed significant growth, reaching 2.77 ± 0.12 cm² and 2.98 ± 0.06 cm² respectively.

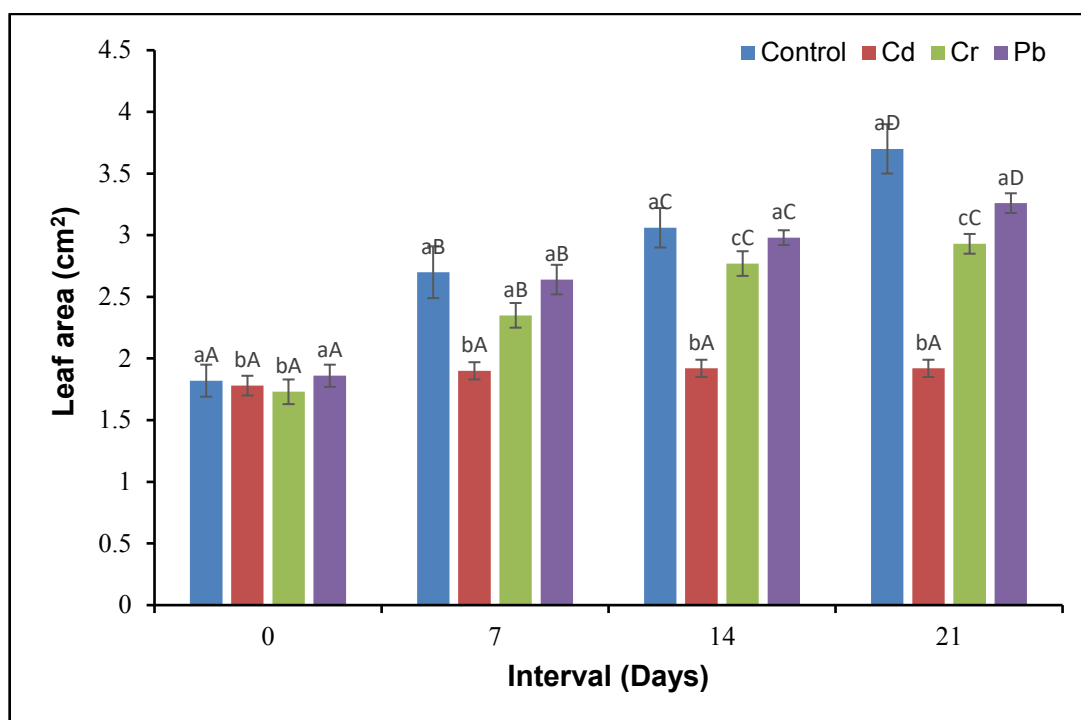


Fig 3.5. Effect of cadmium, chromium and lead treatments on the leaf area of *A. tenella*. Data represent the mean $\pm$ SE of five replicates.

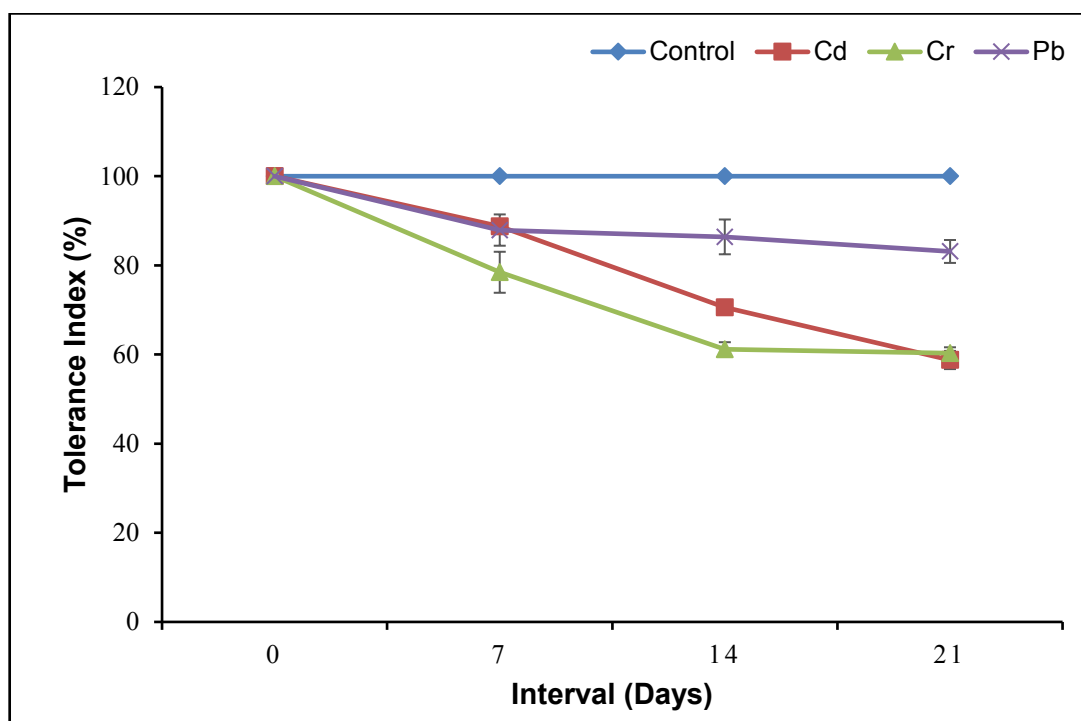


Fig 3.6. Effect of cadmium, chromium and lead treatments on the tolerance index of *A. tenella*. Data represent the mean $\pm$ SE of five replicates.

Cd imparted strong inhibition on the leaf expansion, reaching only to $1.92 \pm 0.07 \text{ cm}^2$ (~7.9% increase). By 21d, control plants showed a twofold increase in the leaf area over the initial size to $3.7 \pm 0.2 \text{ cm}^2$. There was a substantial reduction of 48% and 20.8% respectively in leaf area under Cd and Cr treatments. However, Pb treatment did not cause any significant reduction in leaf area compared to the control ($3.26 \pm 0.08 \text{ cm}^2$) (Fig. 3.5).

The tolerance index in terms of root length was used to measure the tolerance potential of *A. tenella* under metal stress. The tolerance index of Cd- and Cr- treated plants showed a gradual decrease during the treatment period. The tolerance index of 100% at 0d for all treatments serves as the baseline. By 7d, plants exposed to Cd and Pb showed slight reduction to $88.74 \pm 1.31\%$ and $88.74 \pm 1.31\%$ respectively. However, the tolerance index was dropped highly in plants under Cr stress to $77.98 \pm 2.36\%$ indicating early stress effects. On 14d, the index declined further in C and Cr treated plants to $70.61 \pm 1.65\%$ and $61.2 \pm 1.25\%$ respectively, marking a 29.4% and 38.8% decrease from the initial value. Plants under Pb stress maintained a higher index of $86.39 \pm 3.9\%$ (13.6% reduction) after two weeks of treatment. While the tolerance index of Cd-treated plants declined gradually to 58% under prolonged metal exposure, the plants under Pb stress displayed relatively higher tolerance maintaining 83% tolerance index on 21d. Cr consistently showed the most severe effects with a sharp reduction in tolerance to just over 51% by the end of treatment period, which highlights it as the most toxic among the three metals used for treatment (Fig. 3.6).

3.4.2. Anatomical modifications

The SEM analysis of the root, stem and leaves of the control and HM-treated *A. tenella* plantlets on 21d indicates that the treatment with HMs caused significant structural changes especially in the vascular regions and inner pith (Fig 3.7 - 3.10; Table 3.5).

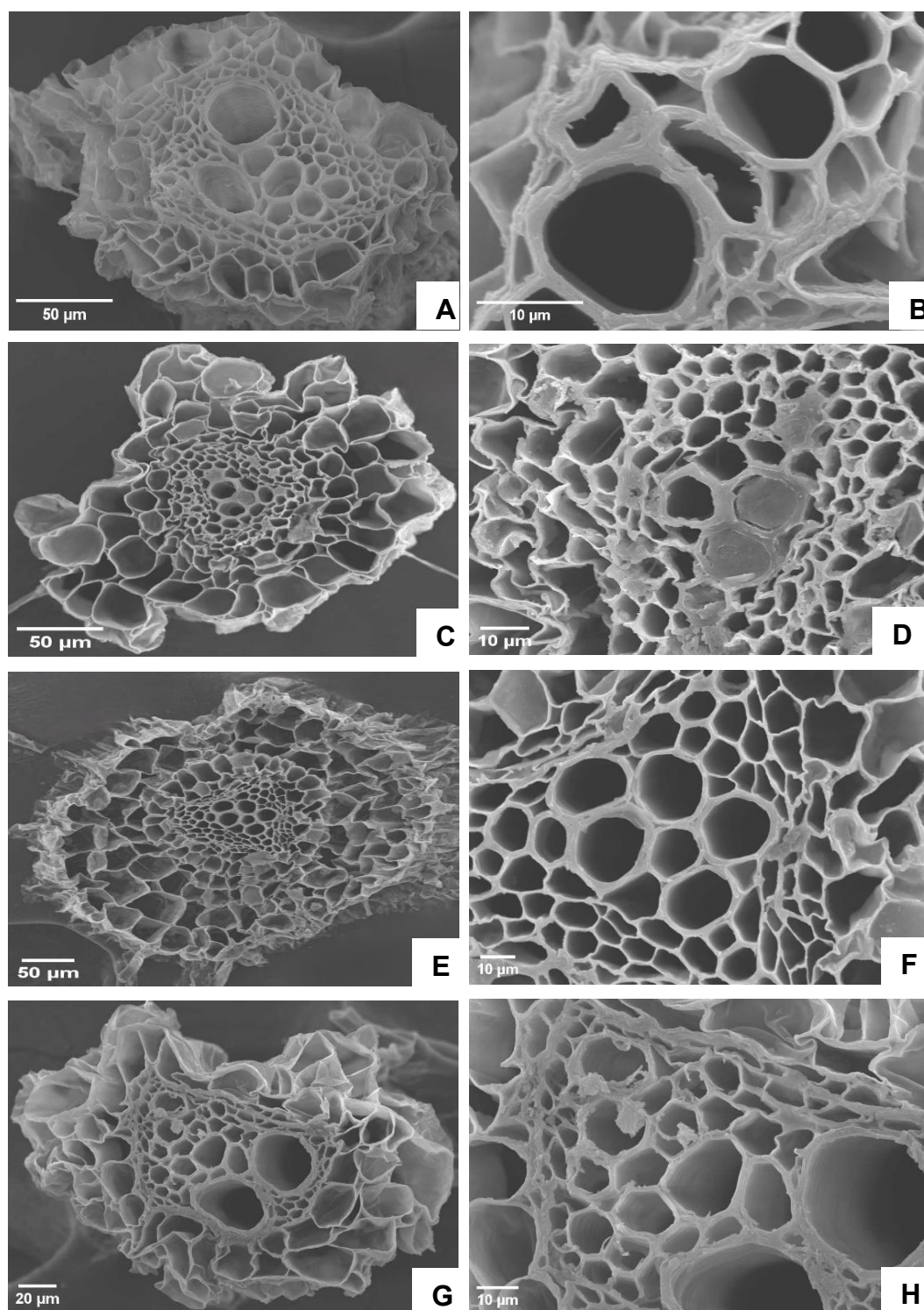


Fig 3.7. Scanning electron micrographs of the ground view and vascular region with inner pith of *A. tenella* root: A & B – control; C & D – Cd-treated; E & F – Cr-treated; G & H – Pb-treated sample.

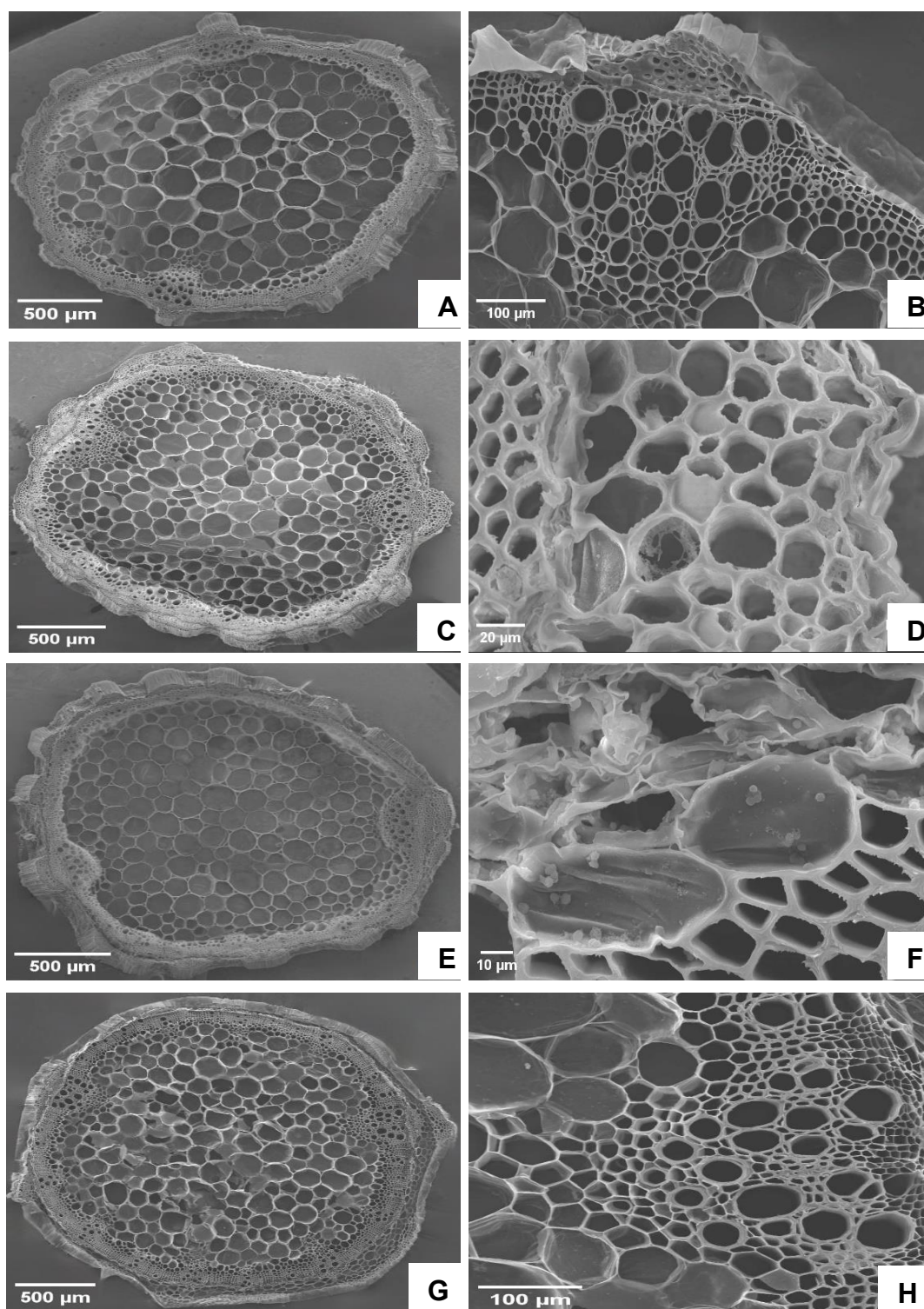


Fig 3.8. Scanning electron micrographs of the ground view and vascular region with inner pith of *A. tenella* stem: A & B – control; C & D – Cd-treated; E & F – Cr-treated; G & H – Pb-treated sample.

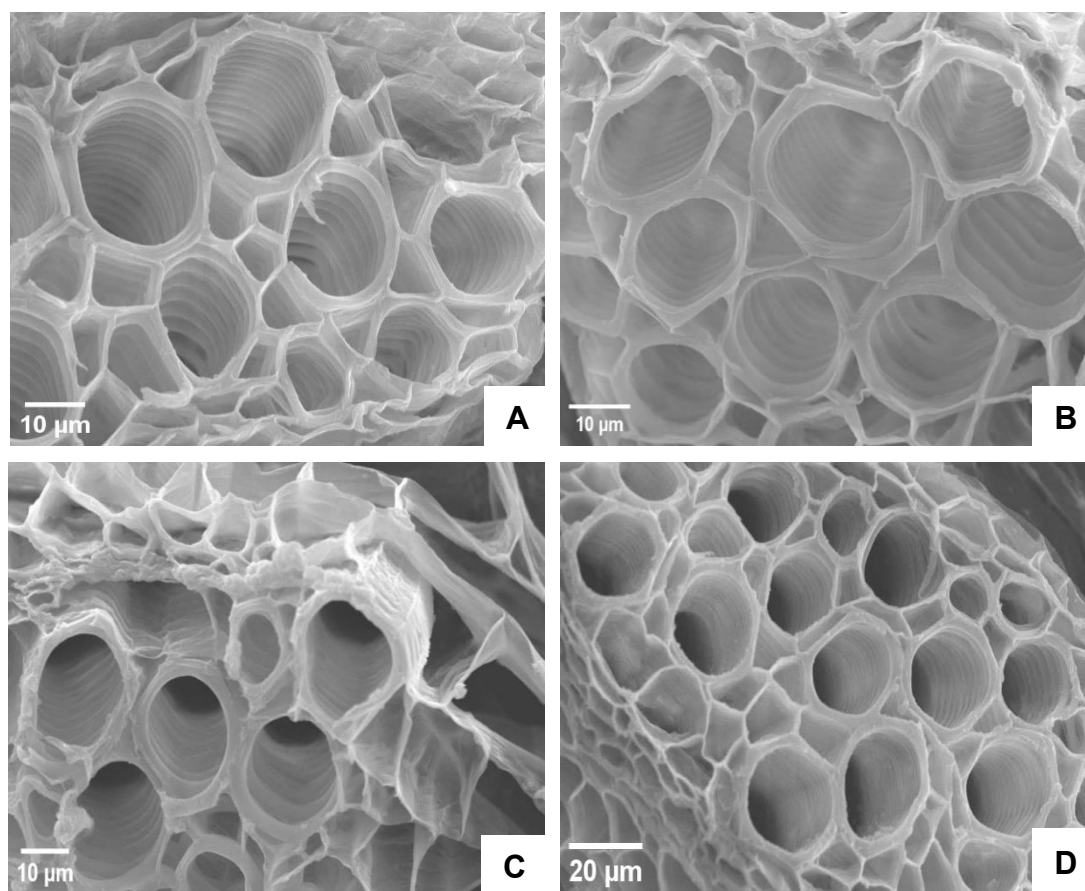


Fig 3.9. Scanning electron micrographs of vascular region of *A. tenella* leaf: A – control; B – Cd-treated; C – Cr-treated; D – Pb-treated sample.

Root. In the cross section of control roots, the epidermal cells, cortical tissue, endodermis, and pericycle remained intact (Fig. 3.7A,B). Metal-treated roots, however, displayed signs of injury including cell distortion. Control roots had a xylem vessel diameter of $12.20 \pm 5.74 \mu\text{m}$ and wall thickness of $1.91 \pm 1.01 \mu\text{m}$ at the final interval of treatment (21d). The diameter was reduced to $9.97 \pm 1.04 \mu\text{m}$ under Cd stress, while the wall thickness increased twofold to $3.22 \pm 0.32 \mu\text{m}$ (Fig. 3.7C,D). Cr stress also caused substantial vascular modifications, with xylem diameter and wall thickness increasing to $13.81 \pm 1.36 \mu\text{m}$ and $3.35 \pm 2.14 \mu\text{m}$ respectively (Fig. 3.7E,F). Under both Cd and Cr treatments, cellular morphology was clearly distorted and dense granules within cortical cells and vascular elements were present. The parenchyma cells in the pith and cortex also showed degeneration. Pb-treated roots had a marked increase in xylem diameter and wall thickness to $20.78 \pm 3.22 \mu\text{m}$ and $4.12 \pm 0.23 \mu\text{m}$ respectively (Fig. 3.7G,H).

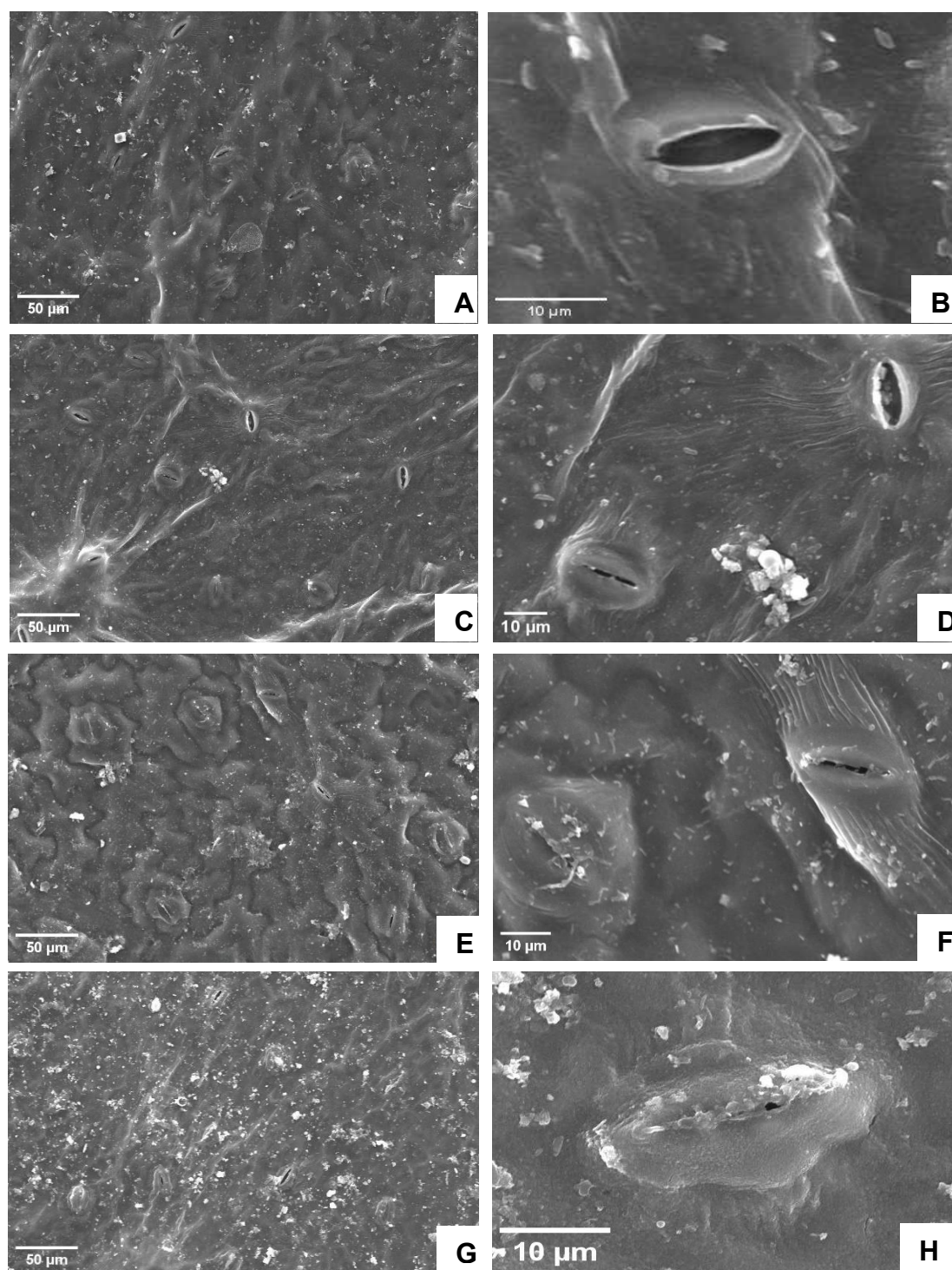


Fig 3.10. Scanning electron micrographs of stomata in *A. tenella* leaf: A & B – control; C & D – Cd-treated; E & F – Cr-treated; G & H – Pb-treated sample.

They displayed cell distortion, loss of cortical cell uniformity, flattening of root shape, and a drastic reduction in cortical cell size. Electron-dense deposits were detected in vacuoles, cell walls, and intercellular spaces of the root tissues, with EDX analysis confirming the presence of Pb in these deposits. These observations indicate that while Cd and Cr primarily induced moderate increases in xylem wall thickness and vessel diameter, Pb stress triggered extensive cellular deformation and degeneration, with heavy deposition of metal within root tissues.

Stem. Control plants recorded a vessel diameter of $37.95 \pm 5.31 \mu\text{m}$ and xylem wall thickness of $5.82 \pm 0.82 \mu\text{m}$ on 21d (Fig. 3.8A,B). Cd treatment induced minimal structural changes, except for the distortions and occlusions in the cortical cells. Xylem anatomical parameters were altered, with a 34% increase in wall thickness ($7.82 \pm 0.46 \mu\text{m}$) and a 24.7% reduction in xylem diameter ($28.58 \pm 2.16 \mu\text{m}$) relative to control (Fig. 3.8C,D). Similar observations were made under Cr stress. The xylem wall thickness increased to $7.64 \pm 0.78 \mu\text{m}$, while the diameter decreased to $27.50 \pm 2.61 \mu\text{m}$. The stem structure showed clear distortion and disintegration of pith cells cell near the vascular region. Dense depositions were observed in these regions and within xylem vessels (Fig. 3.8E,F). However, Pb-treated stems exhibited comparatively less structural alterations. While the tissues and organelles remained intact, pith parenchyma cells appeared torn in certain regions. Pb exposure led to 31% and 24.5% increase in the thickness of xylem walls ($7.64 \pm 0.78 \mu\text{m}$) and xylem diameter ($47.26 \pm 3.44 \mu\text{m}$) respectively compared to the control (Fig. 3.8G,H). Unlike the roots, Pb-treated stems did not show significant cellular distortions or metal depositions.

Leaves. *A. tenella* typically possesses non-glandular trichomes and diacytic shaped stomata scattered over the lamina of leaf surfaces. Stomata in the control leaves were open and fully matured, with well-defined borders and guard cells (Fig.3.10A,B). No major distortions in the overall shape or structure of leaf cells were observed under metal treatments. The xylem diameter and wall thickness in control leaves were $14.30 \pm 6.41 \mu\text{m}$ and $2.24 \pm 0.64 \mu\text{m}$ respectively (Fig. 3.9A). The xylem wall thickness increased significantly in Cd-treated plants ($4.11 \pm 0.26 \mu\text{m}$),

representing 83% rise (Fig. 3.9B). There was only a negligible variation in the vessel diameter ($14.82 \pm 1.27 \mu\text{m}$). Some stomata appeared partially opened, while others were widely opened with thick guard cells and deposits in Cd-treated leaves (Fig. 3.10C,D). The presence of mass depositions in the stomata suggests the possibility for Cd precipitation. The xylem diameter decreased under Cr ($13.65 \pm 0.56 \mu\text{m}$) stress, while the xylem wall thickness showed twofold increase ($4.30 \pm 0.25 \mu\text{m}$) (Fig. 3.9C). The stomata in Cr-treated leaves were partially opened, often with small deposits visible in and around the guard cells (Fig. 3.10E,F). Under both Cd and Cr stresses, aggregates or electron dense depositions were observed within the xylem walls of metal-treated leaves. Also, the number of trichomes was reduced compared to controls, while stomatal density was higher on the abaxial surface of the leaf. Similar to the root and stem, Pb exposure caused an increase in both xylem wall thickness and vessel diameter in leaves. Pb treatment resulted in the highest vessel diameter ($21.92 \pm 0.35 \mu\text{m}$), marking a 53% increase over the control. The xylem wall thickness also reached maximum under Pb ($5.03 \pm 0.47 \mu\text{m}$), reflecting more than twofold increase compared to control (Fig. 3.9D). EDS analysis confirmed the presence of Pb deposits in the vacuoles and cell walls of Pb-treated leaves. Also, the stomata showed variable responses: some remained widely open similar to controls, while others were completely closed (Fig. 3.10G,H).

Table 3.5. The diameter of xylem and thickness of xylem wall of different parts of control and metal-treated *A. tenella* on 21d. Values are the means $\pm$ SE of five biological replicates (n=5).

Sample	Diameter(μm)				Thickness(μm)			
	Control	Cd	Cr	Pb	Control	Cd	Cr	Pb
Root	12.20 ± 5.74	9.97 ± 1.04	13.81 ± 1.36	20.78 ± 3.22	1.91 ± 1.01	3.22 ± 0.32	3.35 ± 2.14	4.12 ± 0.23
Stem	37.95 ± 5.31	28.58 ± 2.16	27.495 ± 2.61	47.26 ± 3.44	5.82 ± 0.82	7.82 ± 0.46	8.49 ± 0.89	7.64 ± 0.78
Leaf	14.30 ± 6.41	14.82 ± 1.27	13.65 ± 0.56	21.92 ± 0.35	2.24 ± 0.64	4.11 ± 0.26	4.3 ± 0.25	5.03 ± 0.47

3.4.3. Element analysis in tissues

EDAX analysis on the three different regions of the plant parts (root, stem and leaf) in control and heavy metal-treated plants showed the significant changes caused by

HMs in the elemental distribution pattern within the plant tissues. The regions selected for the analysis were marked as spectrum 1, spectrum 2, and spectrum 3 respectively for the outer region (epidermis and cortex), a middle region (endodermis and stele) and the inner pith cells.

Root. Carbon (C), Oxygen (O), Potassium (K), Phosphorus (P) and Sulphur (S) were evenly distributed across all regions of both control and Cd-treated roots. However, Cd treatment led to a reduction in C content in the outer and middle regions of the roots, while the inner region remained unaffected.

O distribution decreased in the middle and inner regions of Cd-treated plants but increased by 18% in the outer region. Cd treatment significantly enhanced the levels of K and P in the middle region of roots by 94% each. S content decreased in the outer region of Cd-treated roots but showed slight increases in other regions. Iron (Fe) concentration increased in the inner regions of both samples upon Cd treatment. Magnesium (Mg), Aluminium (Al), and Silicon (Si) were present in the outer region of control roots but were undetectable in any regions of Cd-treated roots. Copper (Cu) and Sodium (Na) were only detected in the roots of Cd-treated plants, showing a reverse trend (Table 3.6; Fig. 3.11A-F).

Carbon, O, K, and P were found to be distributed in both control and Cr treated roots among the various macro-elements. Distribution of C in the three regions of the root reduced in the case of plants subjected to Cr treatments compared to the control. The least reduction in C distribution was observed in the middle region (spectrum 2) followed by the inner region, and the highest reduction was observed in outer region (exodermis and cortex) of roots in plants exposed to Cr treatments. In the plants treated with heavy metals, O content was reduced in the middle area and inner area of the roots, while the O distribution was slightly increased in the outer region of the roots of Cr treated plants compared to control plantlets. The K content was significantly enhanced in the plants exposed to Cr. The maximum increase was observed in the outer region (98%), followed by middle region. The P content in the roots of Cr treated *A. tenella* was also higher in all three regions than the control roots. Calcium (Ca) and S were present in lower quantities in all the

regions of the control roots, whereas they were not detected in roots of Cr treated plantlets. Mg was found only in the outer region. Microelement distribution specifically, Fe, Al and Si were detected in the control roots, Fe in the inner region and Al, Si in the outer region. These elements were not detected in roots of Cr treated plants (Table 3.6; Fig. 3.11A-C,G-I).

Carbon, O, K, P and Ca were evenly distributed across all regions of both control and Pb-treated roots. However, Pb treatment led to a reduction in C and O content in all the regions of the root. While the change in distribution of O was not significant, C got reduced by 5% to 36.7%. Pb treatment significantly enhanced the levels of K and P. P was increased from 1.21% to 11.34%, while K increased from 2.22% to 14.05%. Ca was absent in the outer region of Pb-treated roots but showed slight increases in other regions from control. S, Mg, Al, and Si were present only in control root, while chlorine (Cl) and Na were only detected in the roots of Pb-treated plants. 14.05% Pb was detected in the treated roots, with a higher content on the middle region i.e., 7.91% which indicates that most of the Pb absorbed by *A. tenella* is accumulated in the vascular region of roots (Table 3.6; Fig. 3.11A-C,J-L).

Stem. The number of elements in the stem of control and Cd-treated samples was fewer compared to the leaf and root. The C content increased in all regions of stem exposed to Cd. The distribution of O decreased in the middle region of Cd-treated stem, with the other two regions showing less significant decrease. K was only present in the outer region of treated stem. Ca was solely detected in the inner region of control stem. Cd induced the distribution of Ca in all stem regions but decreased in the inner region. The pattern was reversed for Cu. The Cu content was high in all three regions of control stem, whereas it was only detected in the outer region of Cd-treated plants with a reduced concentration. Zn and S were exclusively present in the stem of control and Cd-treated plants, respectively (Table 3.7; Fig. 3.12A-F).

Cr treatment has not hindered the C and O distribution in all the three regions. The C content showed an increase in the inner region of the Cr treated plants, followed by middle region with reference to stem of control plantlets. But the C content was

slightly reduced in the outer region of the stem of Cr treated plantlets than the control stem. The Cr treated plants had much higher Ca content in the stem, which can be detected in the EDAX profile, and in the control samples, Ca content was detected only in the inner region. Microelements, Cu and Zinc (Zn) were present in the stem of control plantlets, but was not detected in treated samples (Table 3.7; Fig. 3.12A-C,G-I).).

The C content was slightly affected in all regions of stem exposed to Pb. The distribution of O decreased in the all regions of Pb-treated stem. P and K were only present in Pb-treated stem. Ca was detected in the inner region of control stem, but on the outer region of Pb-treated stem. Pb induced an increase in the distribution of Cu and Zn in all root regions. Na was exclusively present in the stem of Pb-treated plants. Pb was not detected in any region of treated stem (Table 3.7; Fig. 3.12A-C,J-L).

Leaf. C, O and K were the macro elements distributed throughout the leaf tissues of both samples (control and Cd-treated). The C content increased in all three regions, with the maximum peak in the inner region by 15%. The distribution of O varied significantly in the control and Cd-treated leaf. While the middle region of the Cd-treated leaf showed a slight decrease in O content, the inner and outer regions exhibited reductions by 4% and 26.5%, respectively, compared to the control. Cd treatment led to a reduction in K content in all regions. Calcium, which was present only in the outer region of control plants, was found to be distributed in all leaf regions exposed to Cd. Cu content increased upon Cd treatment, while levels of P, Na, Zn, and Cl decreased S, Si, and Fe were present only in the Cd-treated leaf (Table 3.8; Fig. 3.13A-F).

Among the several macro-elements, C and O were found to be dispersed evenly and diffusely throughout the leaf tissues in both samples (control and Cr treatments). The C distribution was highly varied in the samples. While the outer region of the leaf in Cr treated plants showed a reduction in C content, the middle region and lower region showed a significant increase compared to control sample. Interestingly, O distribution in the leaf tissues showed a reverse trend. The outer region showed a slight increase in the O content of the leaf in Cr treated plantlets,

whereas the middle region and inner region showed a reduction compared to control sample. Ca content was enhanced by Cr treatment in all the three regions. The K content showed significant reduction in the middle region of the leaf in Cr treated plantlets, followed by the inner region and outer region. In plants exposed to Cr, the S content increased significantly to a level that can be seen in the EDAX profile, and in the control samples, S content was not detected. There was a slight reduction in the P content in the inner region of leaf in Cr treated plantlets than the control. P content was also detected in the outer region of the control stem. Microelement, Cl was detected in the leaf tissues of both the sample but absent in the middle region of the leaf in treated sample. Similarly, Cu was not detected in the outer region of control sample but was present in other regions. The middle region of the leaf of Cr treated plantlet showed a slight decrease in Cu content, while the inner region showed a slight increase. Al was present in the inner region of control stem and middle region of treated sample. Si was detected in the outer and middle regions of treated sample, while it was not detected in control. Na was present in the three regions of control leaf and was not detected in treated sample. Zn content was detected in the middle region of control leaf and in the outer region of Cr treated sample. Fe was also detected after Cr treatment in the outer region of leaf (Table 3.8; Fig. 3.13A-C,G-I).

C, O, P and K were the macro elements distributed throughout the leaf tissues of both control and Pb-treated plants. The C content increased slightly in the middle and inner regions, while O content decreased in the Pb-treated leaf. The inner region of the Pb-treated leaf showed a slight increase in P content. While K content increased in the outer and inner regions on Pb treatment, it was reduced in the middle region. Ca, which was present only in the outer region of control plants, was found to be distributed in the outer and middle leaf regions exposed to Pb. Cu, Cl, Na, and Al were present only in the control leaf. Pb was present in the leaf, with a concentration of 2.02% on the outer region and 0.76% on the middle region (Table 3.8; Fig. 3.13A-C,J-L).

These findings indicate significant alterations in elemental distribution patterns due to heavy metal exposure, highlighting the response of plants to metal toxicity and its potential for phytoremediation.

Table 3.6. SEM-EDX microanalysis data in the roots of *A. tenella*. The macro- and microelements concentrations (% weight) are shown for three different spots, i.e., spectrum 1 (outer region), spectrum 2 (middle region), and spectrum 3 (inner region).

Element	Control			Cd			Cr			Pb		
	1	2	3	1	2	3	1	2	3	1	2	3
Essential macronutrients												
C	63.69	56.04	57.49	55.36	35.48	57.57	35.71	36.96	36.19	40.29	48	54.14
O	33.96	41.75	40.2	41.85	34.52	39.89	37.13	37.9	35.73	37.55	37.24	41.12
K	0.24	1.08	0.31	1.41	19.15	0.61	17.29	15.83	7.76	10.37	2.82	0.83
P	0.32	0.5	0.39	0.63	9.11	0.44	9.86	9.31	5.24	7.78	2.66	0.9
S	0.47	0.26	0.18	0.22	0.35	0.36	-	-	-	-	-	-
Ca	0.31	0.36	0.28	-	-	-	-	-	-	-	0.71	0.45
Mg	0.22	-	-	-	-	-	-	-	-	-	-	-
Essential micronutrients												
Al	0.31	-	-	-	-	-	-	-	-	-	-	-
Si	0.49	-	-	-	-	-	-	-	-	-	-	-
Cu	-	-	-	0.53	0.98	-	-	-	-	-	-	-
Na	-	-	-	-	0.42	-	-	-	-	-	0.35	-
Cl	-	-	-	-	-	-	-	-	-	-	0.31	-
Fe	-	-	0.55	-	-	1.13	-	-	-	0.45	-	-
Non-essential												
Pb	-	-	-	-	-	-	-	-	-	3.56	7.91	2.58

Table 3.7. SEM-EDX microanalysis data in the stems of *A. tenella*. The macro- and microelements concentrations (% weight) are shown for three different spots, i.e., spectrum 1 (outer region), spectrum 2 (middle region), and spectrum 3 (inner region).

Element	Control			Cd			Cr			Pb		
	1	2	3	1	2	3	1	2	3	1	2	3
Essential macronutrients												
C	53.37	52.96	51.67	56.56	59.22	53.06	51.18	55.25	59.61	52.59	52.51	52.19
O	42.72	42.66	46.78	41.65	39.89	46.42	48.39	44.34	40.1	39.74	40.84	39.68
K	-	-	-	0.18	-	-	-	-	-	2.15	2.26	2.87
P	-	-	-	-	-	-	-	-	-	0.38	0.54	1.26
S	-	-	-	0.3	0.43	0.25	-	-	-	-	-	-
Ca	-	-	0.39	0.47	0.46	0.27	0.43	0.41	0.29	0.33	-	-
Essential micronutrients												
Cu	2.58	2.76	1.15	0.87	-	-	-	-	-	4.53	3.14	2.34
Na	-	-	-	-	-	-	-	-	-	-	0.43	-
Zn	1.33	1.62	-	-	-	-	-	-	-	0.29	0.28	1.66

Table 3.8. SEM-EDX microanalysis data in the leaves of *A. tenella*. The macro- and microelements concentrations (% weight) are shown for three different spots, i.e., spectrum 1 (outer region), spectrum 2 (middle region), and spectrum 3 (inner region).

Element	Control			Cd			Cr			Pb		
	1	2	3	1	2	3	1	2	3	1	2	3
Essential macronutrients												
C	56.5	53.42	51.44	59.24	54.91	60.59	49.11	63.66	62.6	56.24	58.29	57.53
O	41.46	40.18	44.06	39.57	42.8	32.38	44.27	34.93	34.69	34.59	36.92	34.87
K	1.24	3.19	2.39	0.4	0.61	0.9	0.35	0.22	0.6	4.13	2.88	5.12
P	-	-	0.56	-	0.28	-	0.43	-	0.25	1.56	0.83	1.87
S	-	-	-	-	0.19	0.19	0.24	0.13	0.47	-	-	-
Ca	0.28	-	-	0.29	0.2	0.77	0.41	0.26	0.28	0.64	0.32	-
Essential micronutrients												
Al	-	-	0.13	-	-	-	-	0.24	-			
Si	-	-	-	-	0.16	0.38	0.67	0.19	-			
Cu	-	0.45	0.58	0.51	0.84	2.3	0.5	0.37	0.87	0.83	-	-
Na	0.39	0.41	0.53	-	-	0.49	-	-	-			
Zn	-	0.8	-	-	-	-	1.47	-	-	-	-	0.61
Cl	0.13	0.67	0.3	-	-	0.83	0.16	-	0.18			
Fe	-	-	-	-	-	1.17	2.05	-	-			
Non-essential												
Pb	-	-	-	-	-	-	-	-	-	2.02	0.76	-

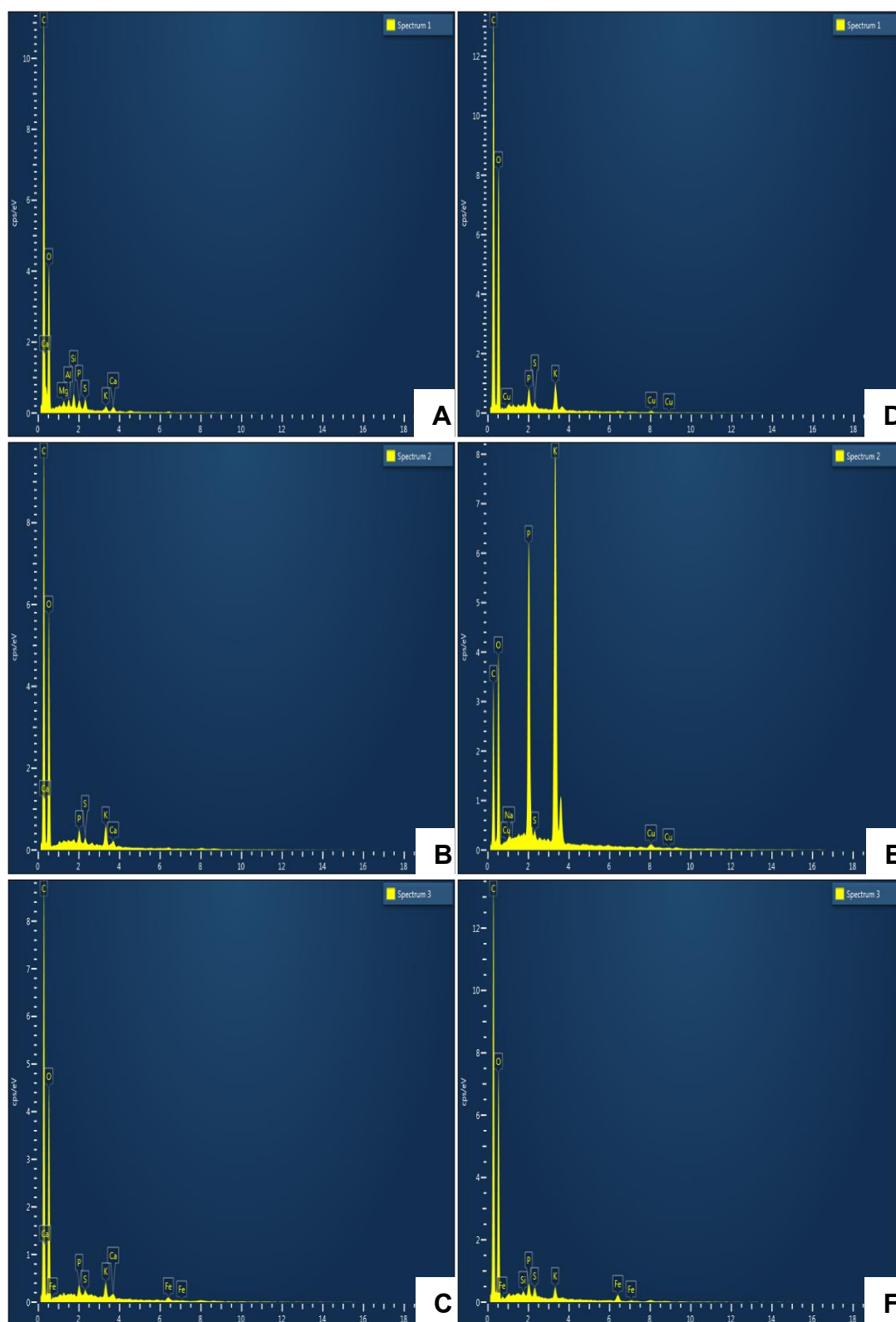


Fig. 3.11. EDX spectra of *A. tenella* roots: spectrum 1 (outer region), spectrum 2 (middle region), and spectrum 3 (inner region). A-C: control; D-F, Cd-treated.

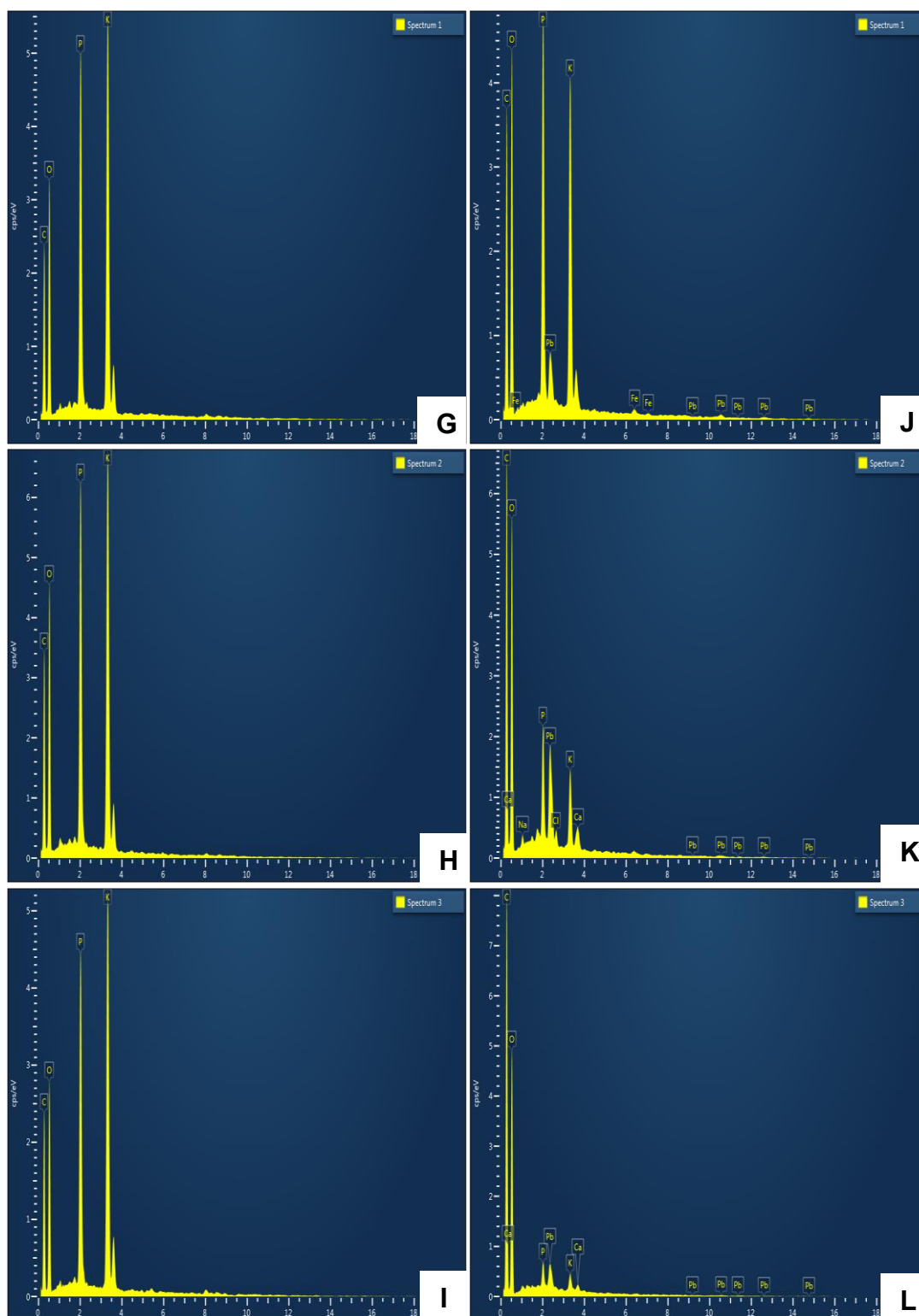


Fig. 3.11. EDX spectra of *A. tenella* roots: spectrum 1 (outer region), spectrum 2 (middle region), and spectrum 3 (inner region). G-I: Cr-treated; J-L, Pb-treated.

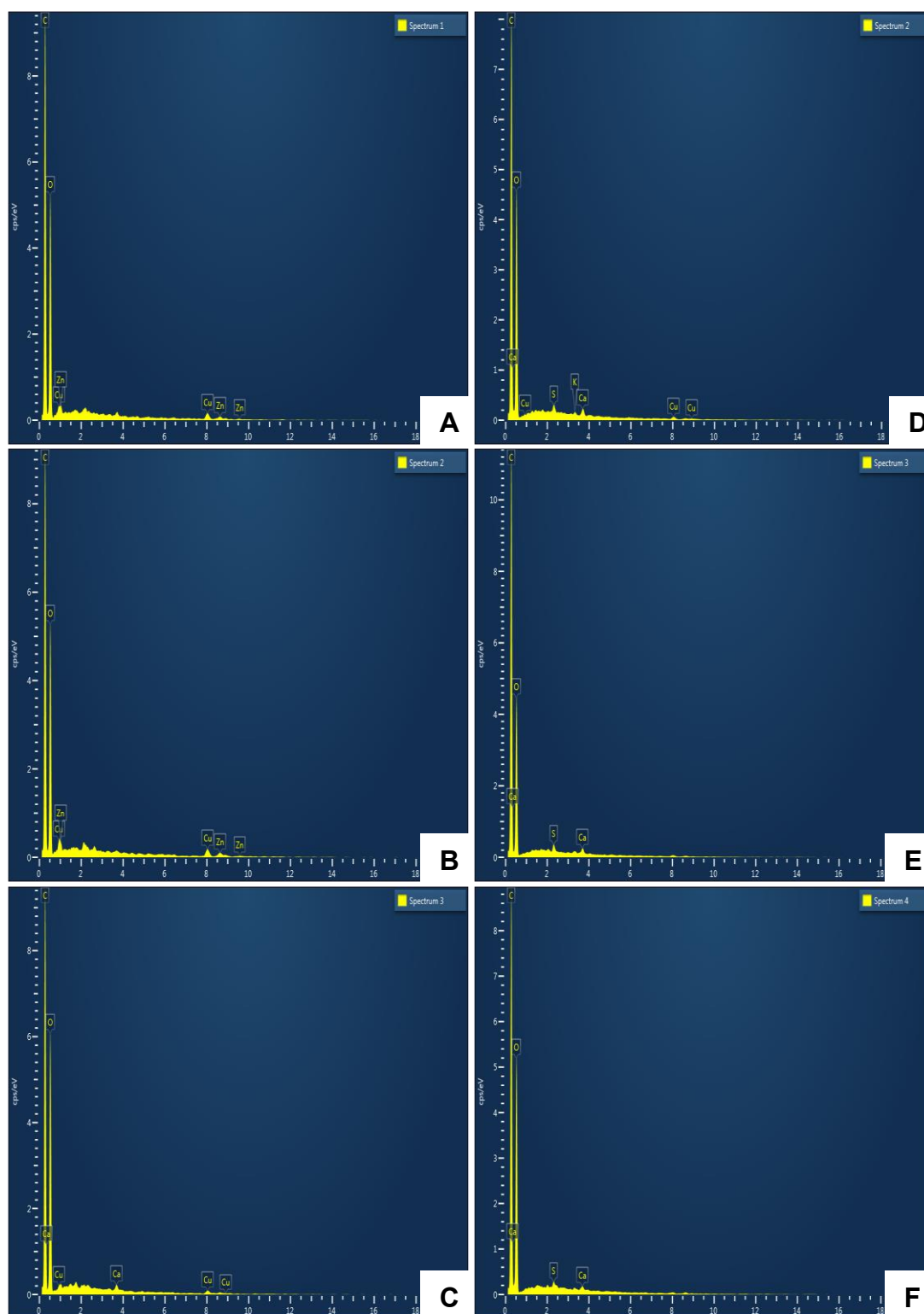


Fig. 3.12. EDX spectra of *A. tenella* stems: spectrum 1 (outer region), spectrum 2 (middle region), and spectrum 3 (inner region). A-C: control; D-F, Cd-treated.

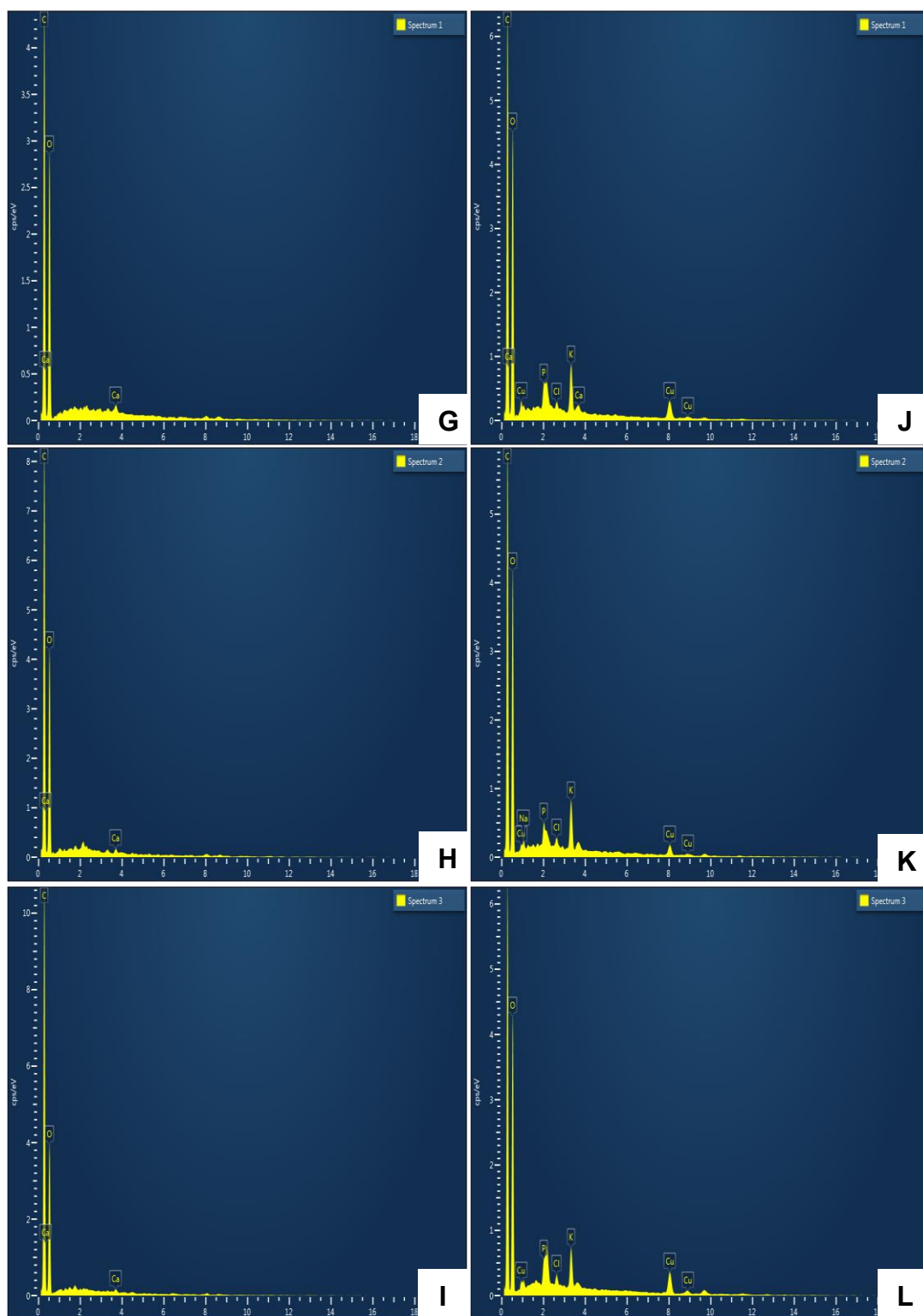


Fig. 3.12. EDX spectra of *A. tenella* stems: spectrum 1 (outer region), spectrum 2 (middle region), and spectrum 3 (inner region). G-I: Cr-treated; J-L, Pb-treated.

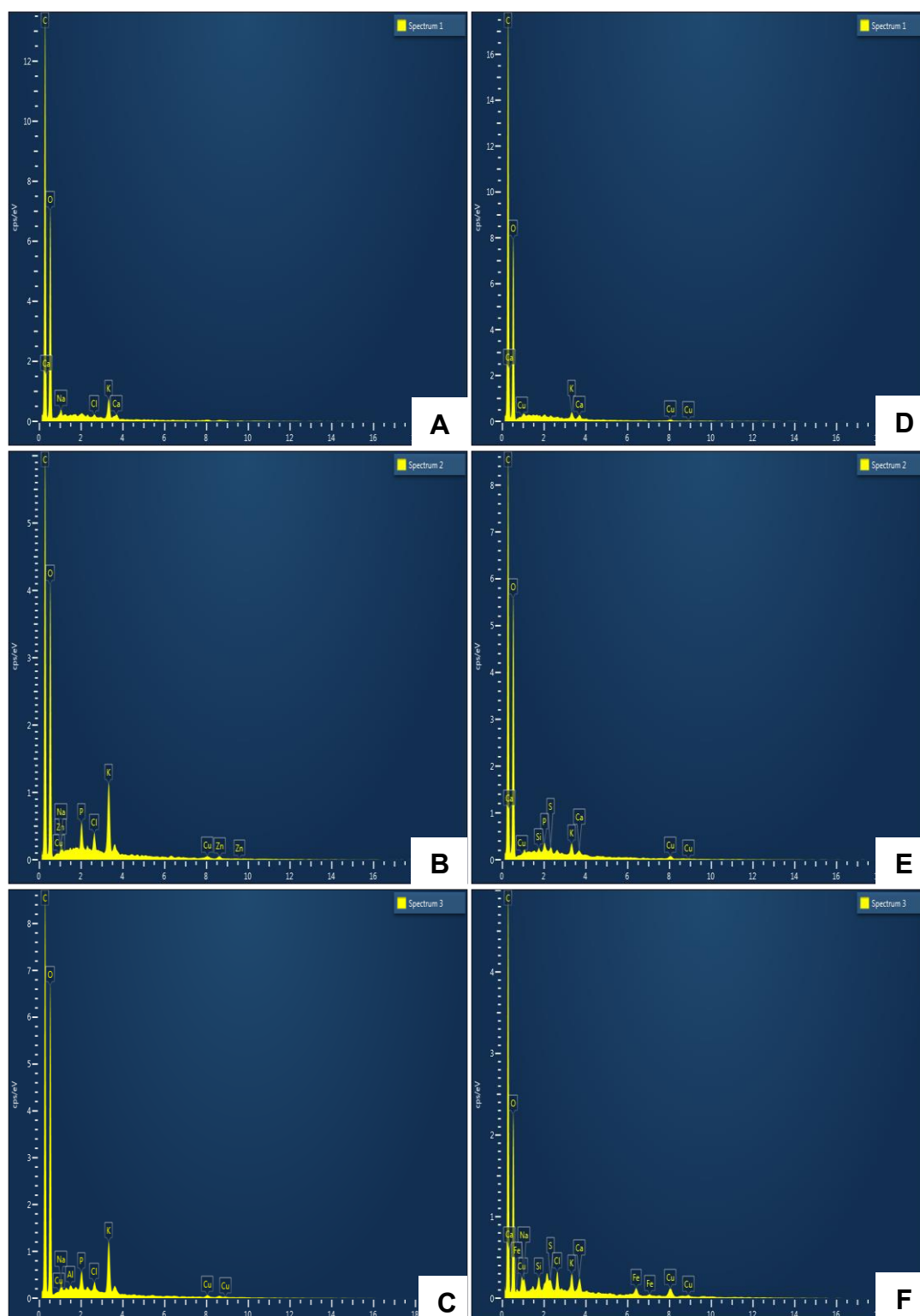


Fig. 3.13. EDX spectra of *A. tenella* leaves: spectrum 1 (outer region), spectrum 2 (middle region), and spectrum 3 (inner region). A-C: Control; D-F, Cd-treated.

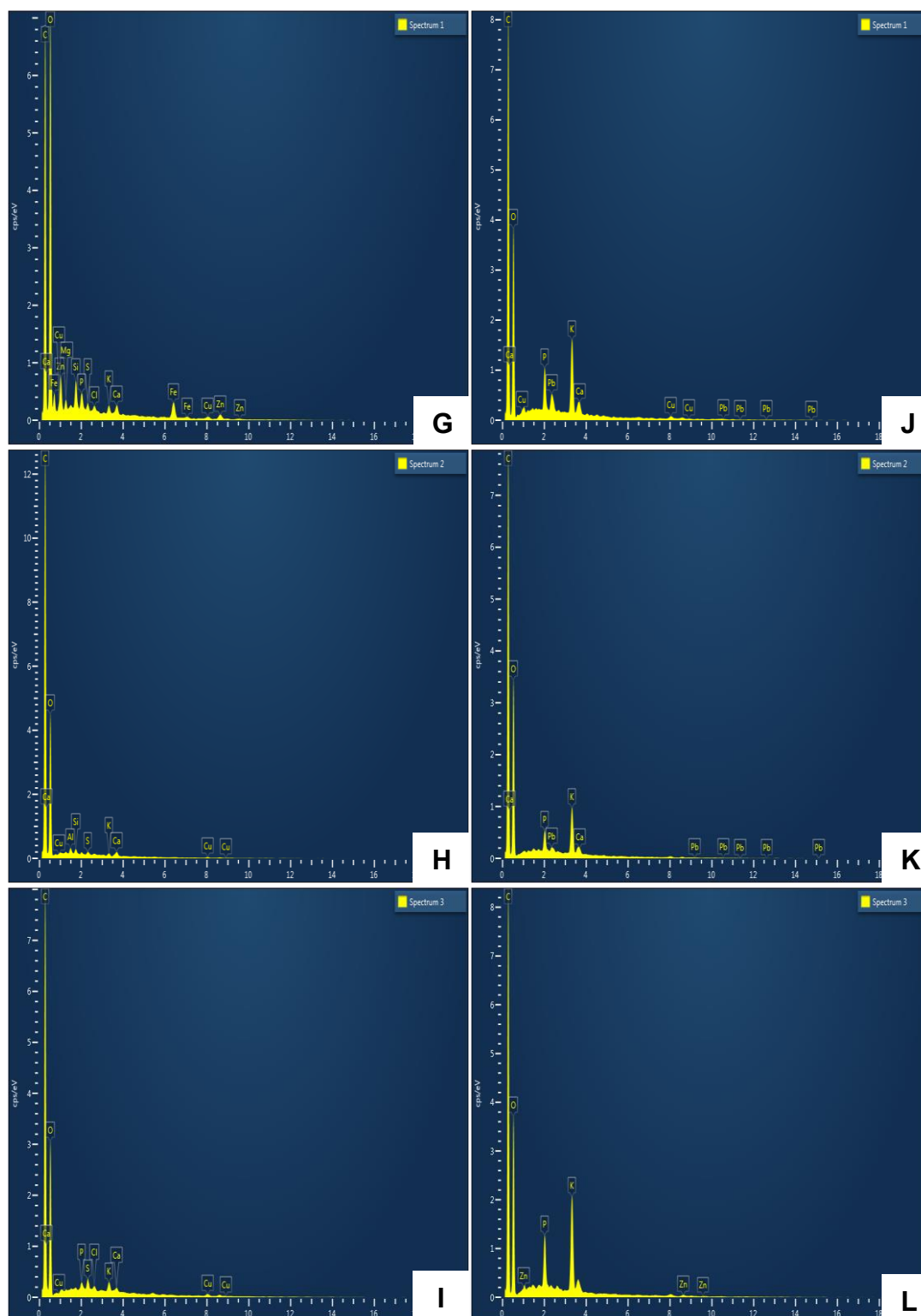


Fig. 3.13. EDX spectra of *A. tenella* leaves: spectrum 1 (outer region), spectrum 2 (middle region), and spectrum 3 (inner region). G-I: Cr-treated; J-L, Pb-treated.

3.4.4. Physiological and biochemical analysis

3.4.4.1. Biomass determination

The changes in shoot and root biomass of *A. tenella* varied under different heavy metal stress over 21d. At 0d, shoot and root biomass was nearly uniform across all treatments, with $\sim 29 \text{ mg kg}^{-1}$ and ~ 7 to 8 mg kg^{-1} respectively. By 7d, control plants showed a significant increase in biomass, with 32.09 ± 0.52 and $9.02 \pm 0.21 \text{ mg kg}^{-1}$ respectively for the shoot and root values. Biomass accumulation was moderate in shoot ($30.48 \pm 0.42 \text{ mg kg}^{-1}$ and $30.11 \pm 0.72 \text{ mg kg}^{-1}$) with comparable root biomass ($\sim 9 \text{ mg kg}^{-1}$ each) under Cd and Cr treatments. Root biomass of Pb-treated plants ($7.89 \pm 0.36 \text{ mg kg}^{-1}$) was slightly reduced compared to Cd and Cr, yet the shoot biomass ($31.41 \pm 0.6 \text{ mg kg}^{-1}$) remained relatively high.

Biomass continued to increase by 14d under all treatments. Control plants showed shoot and root biomass of $35.49 \pm 0.32 \text{ mg kg}^{-1}$ and $11.65 \pm 0.43 \text{ mg kg}^{-1}$ respectively. Cd stress slowed the shoot and root growth by 11.6% and 13.8% respectively, while 13% and 21.8% reduction in shoot and root biomass was observed in Cd-stressed plants compared to the control. However, Pb treatment resulted in comparatively higher biomass in both shoot ($34.95 \pm 0.38 \text{ mg kg}^{-1}$) and root ($10.82 \pm 0.33 \text{ mg kg}^{-1}$) compared to Cd and Cr, and like control levels. By 21d, control plants had the highest biomass values, with $37.94 \pm 0.12 \text{ mg kg}^{-1}$ and $14.08 \pm 0.2 \text{ mg kg}^{-1}$ for shoot and root respectively. 16% and 18% decrease in the shoot biomass was observed specifically under Cd and Cr treatment at the final stage of treatment over the control plants. Root biomass exhibited a consistent decrease under Cd stress, although no significant differences were found among treated plants at this specific interval ($P < 0.05$). There was a drastic reduction in the root biomass (33%) on 21d in Cr-treated plants compared to the control. Pb-treated plants closely followed control values (Fig. 3.14A,B).

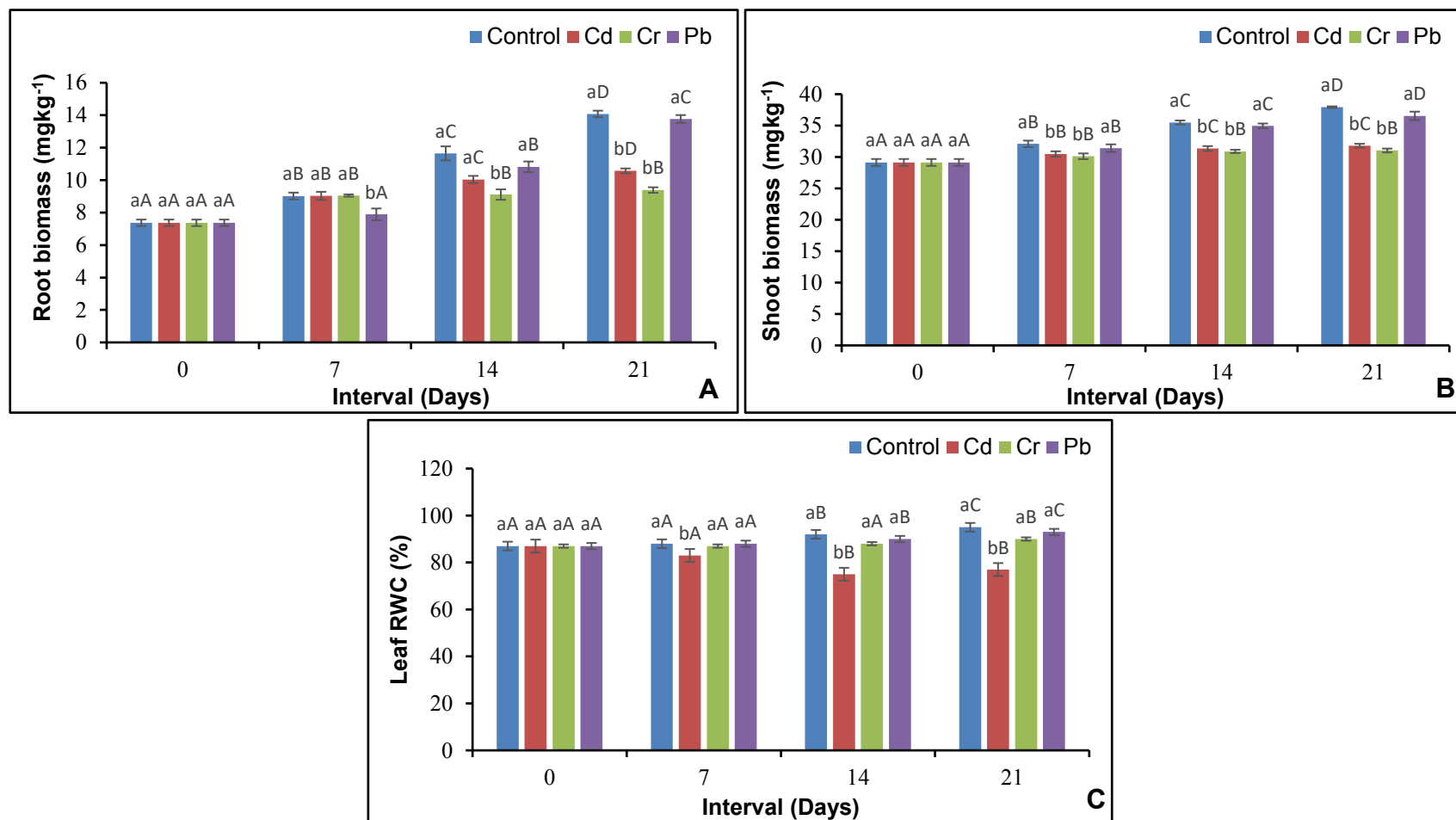


Fig 3.14. Effect of cadmium, chromium and lead on the biomass (A & B) and leaf RWC (C) in *A. tenella*. Data represent the mean $\pm$ SE of five replicates.

3.4.4.2. Relative water content (RWC)

Heavy metal treatments reduced the relative water content (RWC) of leaves in *A. tenella* compared to control plants. RWC in control plants increased slightly from $87 \pm 0.71\%$ to $88 \pm 1.38\%$ by 7d. Cd-treated plants showed a moderate reduction to $83 \pm 0.71\%$ indicating the early signs of water imbalance. While plants under Cr stress showed maintained their RWC at $87 \pm 1.1\%$. Pb-treated plants showed an increase in RWC to $88 \pm 0.89\%$, like control. On 14d, control plants showed a 5% increase in RWC. Cd stress caused a drastic drop to $75 \pm 1.09\%$, indicating water deficit stress and reduced tissue hydration. Cr-treated plants maintained a stable RWC as earlier, while Pb-treated plants showed a slight increase by 3.4% indicating good water retention despite metal stress. The control group reached the highest RWC on 21d at $95 \pm 0.71\%$. Cd-treated plants showed a slight recovery of 2.6% ($77 \pm 0.71\%$). Pb and Cr treatments maintained relatively stable water status comparable to control at this interval. So, it was noted that Cd stress affected the water balance in *A. tenella* more adversely leading to a marked reduction in leaf RWC (Fig. 3.14C).

3.4.4.3. Photosynthetic efficiency of the leaf

Photosynthetic pigment content, including chlorophyll a (Chl a), chlorophyll b (Chl b), carotenes and total chlorophyll (total Chl) was measured in leaves of *A. tenella* subjected to Cd, Cr and Pb stresses at various intervals of 0, 7, 14, and 21 days. The results showed significant alterations in pigment accumulation under different metal treatments.

In control plants, Chl a content increased steadily from $0.4 \text{ mg g}^{-1} \text{ FW}$ at 0d to 1.89 mg g^{-1} at 21d, indicating a 4.73-fold increase. Cd-treated plants showed a modest but gradual increase of 2.3-fold over the initial value, reaching 0.92 mg g^{-1} at 21d. However, this reflected a 51% decrease in Chl a content over the control at the final interval. Chl a consistently decreased under Cr treatment reaching 0.24 mg g^{-1} by 21d, marking 87% decrease over the control value which indicated the severe inhibition of Chl a synthesis under Cr stress. In contrast to Cd and Cr, Pb-treated plants maintained a progressive increase till 21d, peaking at 1.52 mg g^{-1} with a 3.8-fold increase over the initial value, closely resembling the control trend (Fig. 3.15A).

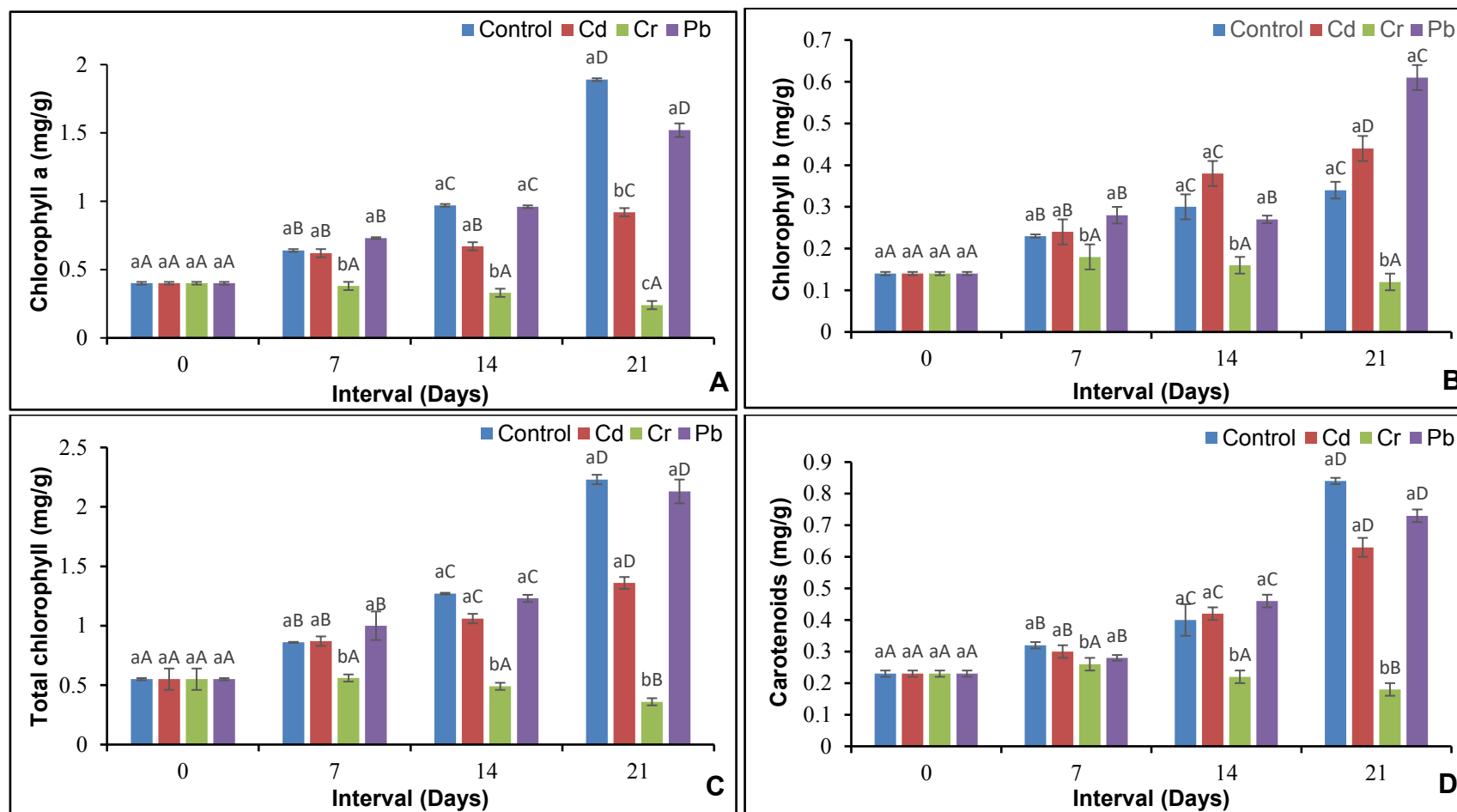


Fig 3.15. Effect of cadmium, chromium and lead on chlorophyll and carotenoid contents in *A. tenella* leaf. Data represent the mean $\pm$ SE of five replicates.

The Chl b content in control plants increased gradually from 0.14 mg g⁻¹ FW at 0d to 0.34 mg g⁻¹ by 21d. While Cd- and Pb-treated plants showed a consistent increase up to 21d with a peak of 0.44 mg g⁻¹ and 0.61 mg g⁻¹ FW respectively, surpassing the control at the final interval. However, Cr stress caused a continuous reduction reaching as low as 0.12 mg g⁻¹ at 21d, a 14% and 64.7% reduction respectively from the initial value and the control (Fig. 3.15B).

The total chlorophyll content followed the same trend as Chl a in all treatments. Control plants exhibited a steady rise from 0.53 mg g⁻¹ FW at 0d to 2.23 mg g⁻¹ at 21d. Cd treatment resulted in an initial increase up to 14d (1.06 mg g⁻¹ FW) and a marginal rise to 1.36 mg g⁻¹ at 21d, indicating a 2.56-fold increase over the initial value. Cr stress led to a consistent decline throughout the treatment period, with the lowest value of 0.36 mg g⁻¹ on 21d, highlighting the strong inhibitory effect of Cr on chlorophyll synthesis. Pb-treated plants showed a significant fourfold increase in total chlorophyll, reaching 2.13 mg g⁻¹ at 21d which was almost comparable to the control (Fig. 3.15C).

Carotene levels in control plants increased from 0.23 mg g⁻¹ at 0d to 0.84 mg g⁻¹ FW by 21d, implying a 3.65-fold increase over the initial value. Cd- and Pb-treatment caused a gradual increase throughout the treatment period reaching a peak at 21d with 0.63 mg g⁻¹ and 0.73 mg g⁻¹ respectively. Cr stress led to a consistent decline, reducing carotenes to 0.18 mg g⁻¹ (22% from the initial value) (Fig. 3.15D).

3.4.4.4. Total Protein content

Protein content in root, stem and leaf tissues of *A. tenella* was quantified under metal stress. Control plants exhibited consistent increases in protein content throughout the treatment period, while metal treatments caused alterations depending on stress and tissue.

Control roots showed a 3.5-fold increase in protein content from 2.18 mg g⁻¹ FW at 0d to 7.65 mg g⁻¹ at 21d. Cd-treated roots showed 78% increase in protein content on 7d, when it peaked at 3.89 mg g⁻¹, but later declined to 2.21 mg g⁻¹ by 21d. Cr stress also followed a similar pattern. The protein content in Cr-treated roots

increased initially to 4.89 mg g⁻¹ on 7d, and then declined to 2.41 mg g⁻¹ at 21d. Pb treatment also induced a steady increase to 4.65 mg g⁻¹ at 14d and maintained 4.04 mg g⁻¹ by 21d (Fig. 3.16A).

The protein content in control stem increased from 3.82 mg g⁻¹ FW at 0d to 12.98 mg g⁻¹ at 21d. Cd stress induced early protein accumulation, peaking at 8.6 mg g⁻¹ at 14d, but later dropped to 3.28 mg g⁻¹ by 21d. Cr stress caused a rapid decrease from 7d (6.9 mg g⁻¹) to 21d (2.51 mg g⁻¹). Similar pattern to Cd was observed in protein content of Pb-treated stem. It declined to 5.95 mg g⁻¹ on 21d after an initial increase at 7.54 mg g⁻¹ on 14d (Fig. 3.16B).

Leaves had the highest protein content across tissues. Control leaves showed a 3.21-fold increase from 0d (7.4 mg g⁻¹ FW) to 21d (23.76 mg g⁻¹). Cd- and Cr-treatments enhanced the protein content in leaves till 14d, but later got reduced by 37% (10.18 mg g⁻¹) and 29% (9.58 mg g⁻¹) respectively. The protein content in Pb-treated leaves rose steadily to 19.87 mg g⁻¹ by 21d, marking a 2.7-fold increase over the initial value, comparable to control (Fig. 3.16C).

3.4.4.5. Soluble sugars

In control roots, soluble sugar levels increased 2.66-fold from 1.21 mg g⁻¹ FW at 0d to 3.22 mg g⁻¹ at 14d and later declined to 2.17 mg g⁻¹ at 21d. Cd treatment caused a marked reduction of 35% in sugar content of roots by 7d to 0.79 mg g⁻¹. It was partially recovered by 14d to 1.33 mg g⁻¹, but again dropped by 12% on 21d to 1.06 mg g⁻¹. Cr stress initially caused a 56% increase in sugar levels at 7d (1.89 mg g⁻¹), but later declined gradually to reach 0.91 by 21d. In contrast, Pb-treated roots showed consistent increase in sugars, peaking at 2.87 mg g⁻¹ on 14d and maintained at 2.43 mg g⁻¹ at 21d (Fig. 3.17A).

The control stem exhibited a steady rise in sugar content from 3.09 mg g⁻¹ FW at 0d to 9.71 mg g⁻¹ at 21d, representing a 3.14-fold increase over the experimental period. Cd-treated stems showed a rapid increase at 7d to 7.86 mg g⁻¹ and maintained high levels at 14d at 6.89 mg g⁻¹, but later dropped to 4.99 mg g⁻¹ by 21d. Cr stress also had a similar effect on sugar content of stem. It rose modestly through 14d (6.61 mg

g^{-1}) before falling to 5.01 mg g^{-1} by 21d. The sugar levels peaked at 7d under Pb stress (8.08 mg g^{-1}) with 22.6% increase over the control, followed by a decline to 5.41 mg g^{-1} by 21d (Fig. 3.17B).

Control leaves showed a gradual and consistent increase in sugar content, from 5.9 mg g^{-1} FW to 10.47 mg g^{-1} at 21d, marking a 78% rise. Cd stress led to a moderate increase of 39% up to 14d (8.18 mg g^{-1}), followed by a sharp decline by 21d (6.9 mg g^{-1}). Cr-treated leaves showed an early peak at 7d with 36% increase (8.04 mg g^{-1}), but sugar levels declined to nearly 5.97 mg g^{-1} by 21d. Pb exposure led to higher sugar accumulation in leaves up to 14d by 75% increase (10.34 mg g^{-1}), although levels dropped by 18% to 6.97 mg g^{-1} by 21d (Fig. 3.17C).

3.4.4.6. Proline

The accumulation of proline, a key stress-responsive metabolite, was significantly influenced under metal treatments in *A. tenella*. *A. tenella* exhibited a strong and progressive increase in proline accumulation under all heavy metal stresses, especially in leaves and stems.

The control plants exhibited a gradual decline in proline levels of roots from 5.21 mg g^{-1} FW at 0d to 2.23 mg g^{-1} by 21d, indicating a 57% reduction. Metal treatments induced the enhancement of proline production. The proline content in Cd-treated roots rose to 8.05 mg g^{-1} at 7d and further to 14.06 mg g^{-1} by 21d, reflecting a sixfold increase over the control. Cr stress resulted in a similar pattern, with proline levels rising 3.12-fold to 16.24 mg g^{-1} by 21d. Pb exposure induced the highest proline accumulation in roots (8.47-fold), reaching 44.1 mg g^{-1} by 21d (Fig. 3.18A).

The control stems showed minor fluctuations in proline content, peaking at 2.62 mg g^{-1} FW at 14d and then decreasing to 1.73 mg g^{-1} at 21d. Cd- and Cr-treatments led to a rapid and substantial increase in proline content of ~16-fold, reaching 38.08 mg g^{-1} and 37.7 mg g^{-1} respectively. Comparatively, Pb stress stimulated less proline accumulation with levels reaching 14.54 mg g^{-1} at the final interval, still higher than control by 8.4-fold (Fig. 3.18B).

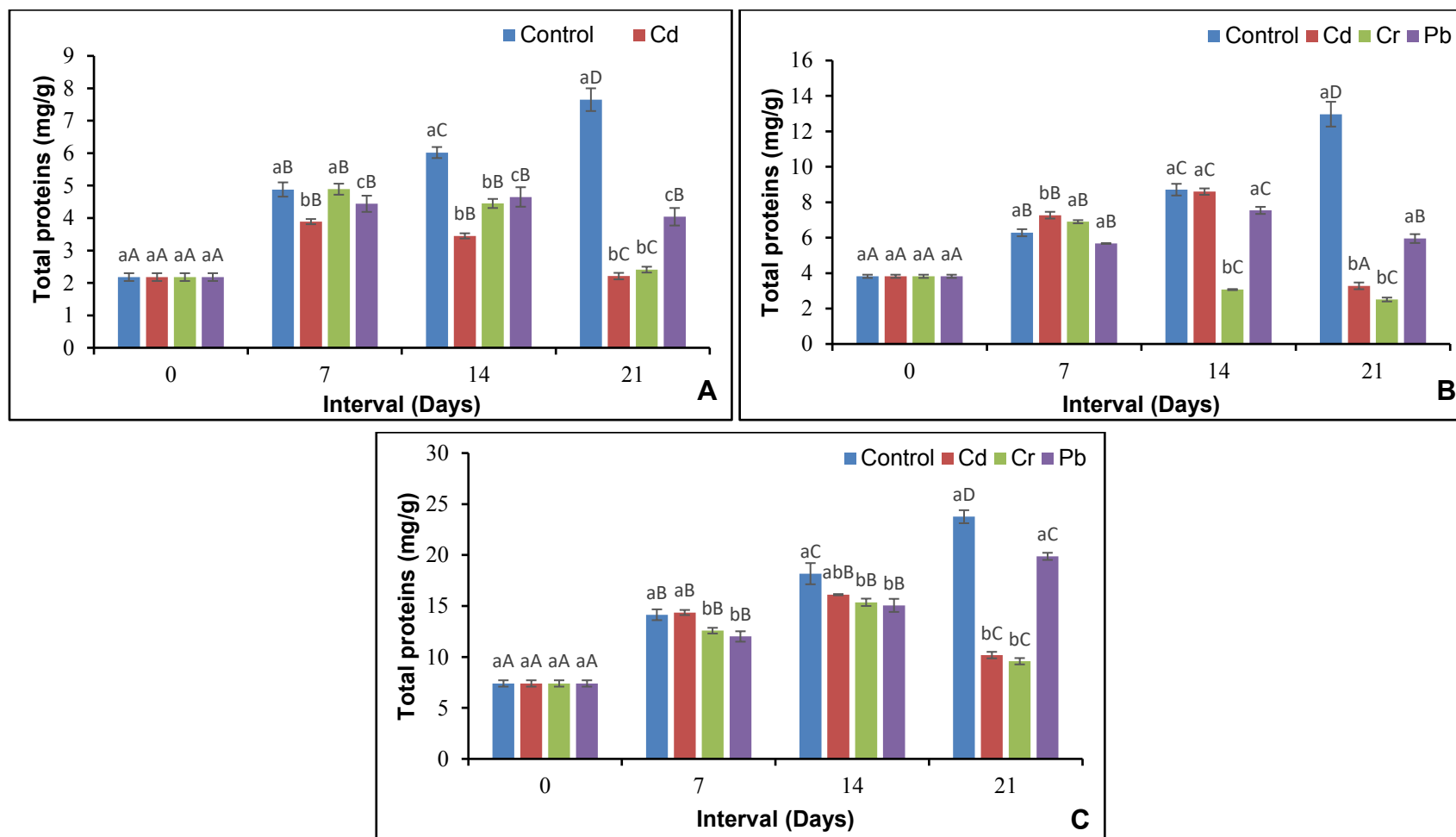


Fig 3.16. Effect of cadmium, chromium and lead on total protein content in *A. tenella*: A. Root, B. Stem, C. Leaf. Data represent the mean $\pm$ SE of five replicates.

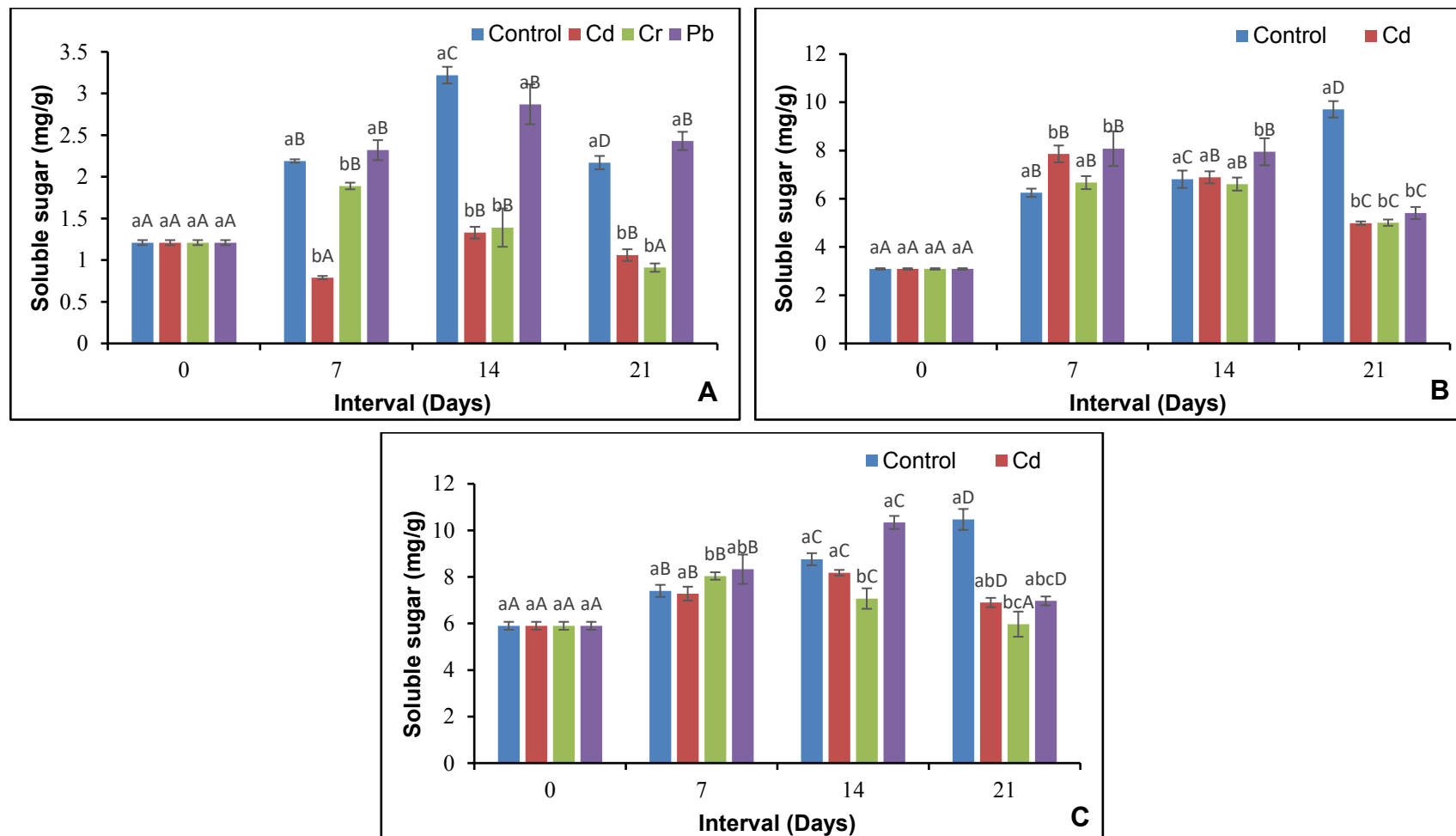


Fig 3.17. Effect of cadmium, chromium and lead on soluble sugar content in *A. tenella*: A. Root, B. Stem, C. Leaf. Data represent the mean $\pm$ SE of five replicates.

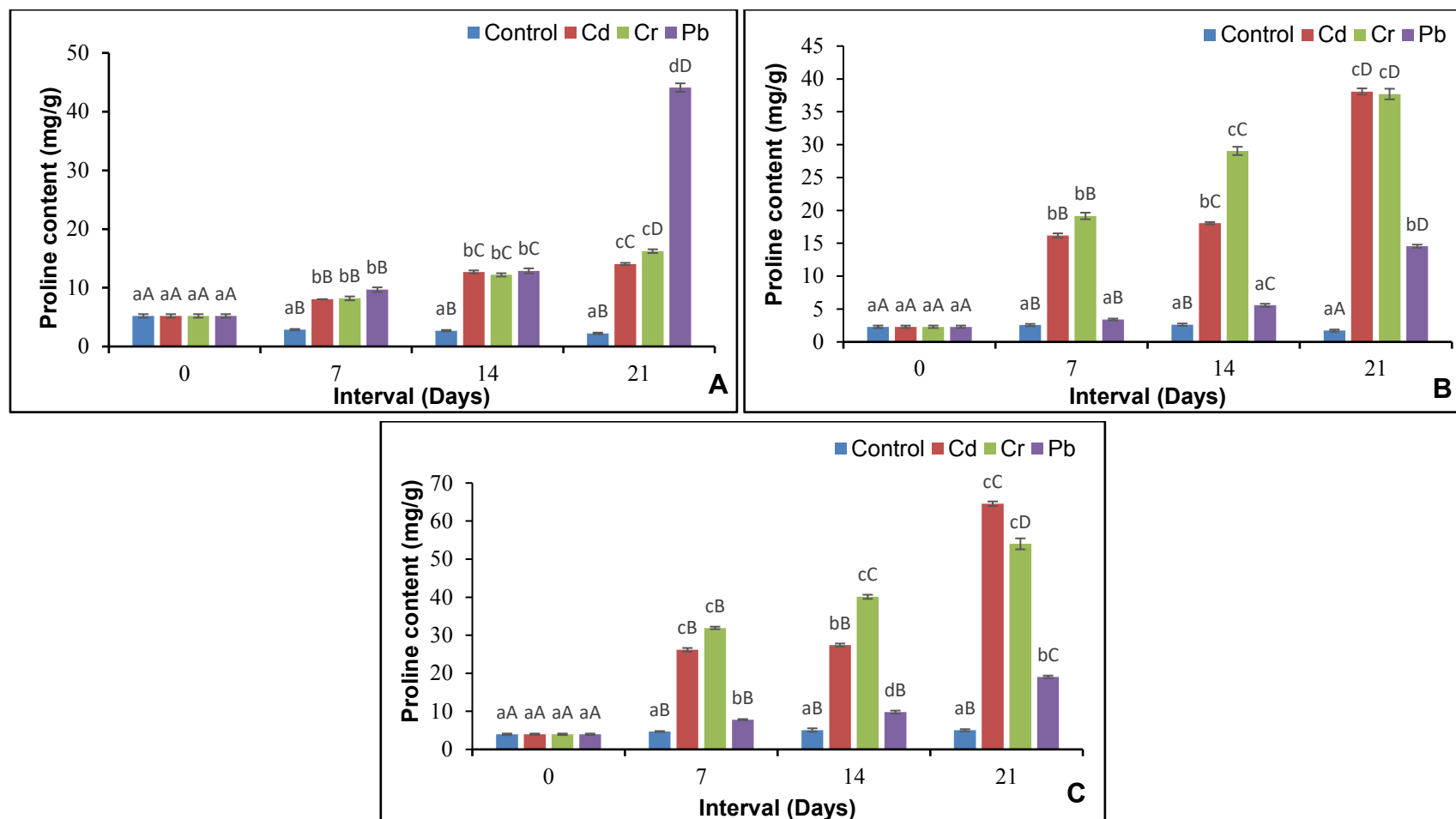


Fig 3.18. Effect of cadmium, chromium and lead on proline content in *A. tenella*: A. Root, B. Stem, C. Leaf. Data represent the mean $\pm$ SE of five replicates.

The leaves exhibited the higher enhancement in proline accumulation. Control plants displayed a modest increase from 3.97 mg g⁻¹ FW at 0d to 5.03 mg g⁻¹ at 21d. Cd-treated leaves showed a sharp increase in proline levels reaching 64.55 mg g⁻¹ by 21d (16.3-fold). Cr-treated leaves followed a similar trend, accumulating 54.01 mg g⁻¹ proline at 21d representing a 13.6-fold increase. Pb exposure induced a more moderate response in leaves, with proline increasing to 19.07 mg g⁻¹ by 21d (Fig. 3.18C).

3.4.4.7. Total phenolics

Phenolics showed dynamic changes depending on the type of metal, tissue, and duration of exposure in *A. tenella*.

In the roots, control plants showed slight fluctuations in phenolic content, ranging from 0.21 mg g⁻¹ FW at 0d to a marginally decreased level of 0.17 mg g⁻¹ by 21d. Cd-treated roots slightly declined to 0.26 mg g⁻¹ at 21d after displaying a marked increase of 2.43-fold peaking at 0.51 mg g⁻¹ at 14d. Cr stress also elevated root phenolic content, reaching 0.44 mg g⁻¹ at 14d and maintaining 0.37 mg g⁻¹ at 21d (76% increase from the initial value). Pb treatment resulted in a consistent increase, with levels rising to 0.51 mg g⁻¹ at 21d marking a 2.43-fold increase over the initial value (Fig. 3.19A).

The control plants exhibited a small rise in phenolic content of stems till 14d (0.39 mg g⁻¹ FW) followed by a decrease to 0.13 mg g⁻¹ by 21d. Cd treatment induced significant enhancement in phenolic accumulation of 4.5-fold, increasing from 0.1 mg g⁻¹ at 0d to 0.45 mg g⁻¹ at 21d. Cr stress showed a similar trend. The stem phenolics under Cr exposure reached 0.49 mg g⁻¹ at 21d, showing a 4.3-fold increase over the initial value. Pb-treated stems exhibited a lower value compared those under Cd and Cr between days 7 and 14 but ended up with a moderate increase of 4.3-fold at 21d (0.43 mg g⁻¹) (Fig. 3.19B).

The control leaves showed a gradual rise in phenolic content from 0.34 mg g⁻¹ FW at 0d to 0.71 mg g⁻¹ at 14d, followed a slight reduction to 0.54 mg g⁻¹ at 21d. Cd stress caused the highest phenolic accumulation at 14d (0.86 mg g⁻¹) with 2.5-fold

increase over the initial value, before dropping to 0.54 mg g^{-1} by 21d. Cr treatment induced a sustained increase in leaf phenols up to 14d, reaching 0.76 mg g^{-1} and maintained at 0.68 mg g^{-1} at 21d (twofold increase over the initial value). Pb stress caused a more subdued increase, peaking at 7d (0.59 mg g^{-1}) and declining to 0.41 mg g^{-1} at 21d (Fig. 3.19C).

These observations highlight the dynamic modulation of phenolic metabolism in *A. tenella* as an important part of its stress tolerance strategy.

3.4.4.8. Flavonoids

Heavy metal treatments induced significant variation in flavonoid accumulation in *A. tenella* tissues across time points and treatments, reflecting its vital role in antioxidant defence and stress adaptation. All metal treatments induced a steady and sharp increase in flavonoids throughout the treatment period compared to control, with Cr showing the most prominent effect.

Control plants exhibited minor fluctuations in flavonoid levels of roots, from 0.12 mg g^{-1} FW at 0d to 0.5 mg g^{-1} at the final interval. Cd-treated roots showed a steep increase of 12.58-fold, reaching 1.51 mg g^{-1} at 21d. Cr and Pb stress also induced a similar enhancement, with flavonoid content rising to 1.23 mg g^{-1} and 0.97 mg g^{-1} by 21d, marking a 10.25-fold and 8.08-fold increase respectively (Fig. 3.20A).

The control stems maintained relatively stable flavonoid levels, increasing modestly from 8.6 mg g^{-1} FW at 0d to 9.46 mg g^{-1} by 21d. Cd and Cr treatment slightly elevated the stem flavonoid content to nearly 13 mg g^{-1} each, marking a ~1.5-fold increase by 21d. Pb exposure led to a moderate rise in flavonoids till 21d, ending up at 11.88 mg g^{-1} (1.38-fold increase over initial value) (Fig. 20B).

Leaf tissues generally showed the highest levels of flavonoids across all treatments. The leaves of control plants displayed a slow increase from 18.72 mg g^{-1} to 21.45 mg g^{-1} over the treatment period. Cd stress significantly enhanced flavonoid accumulation in leaves, ending up with a 72.7% increase to 32.33 mg g^{-1} at 21d. Cr-treated leaves also exhibited higher flavonoid levels in leaves with 1.86-fold increase, peaking at 34.89 mg g^{-1} . Pb exposure also induced a moderate but consistent rise to 30.27 mg g^{-1} by 21d, reflecting a 61.7% increase over the initial concentration (Fig. 3.20C).

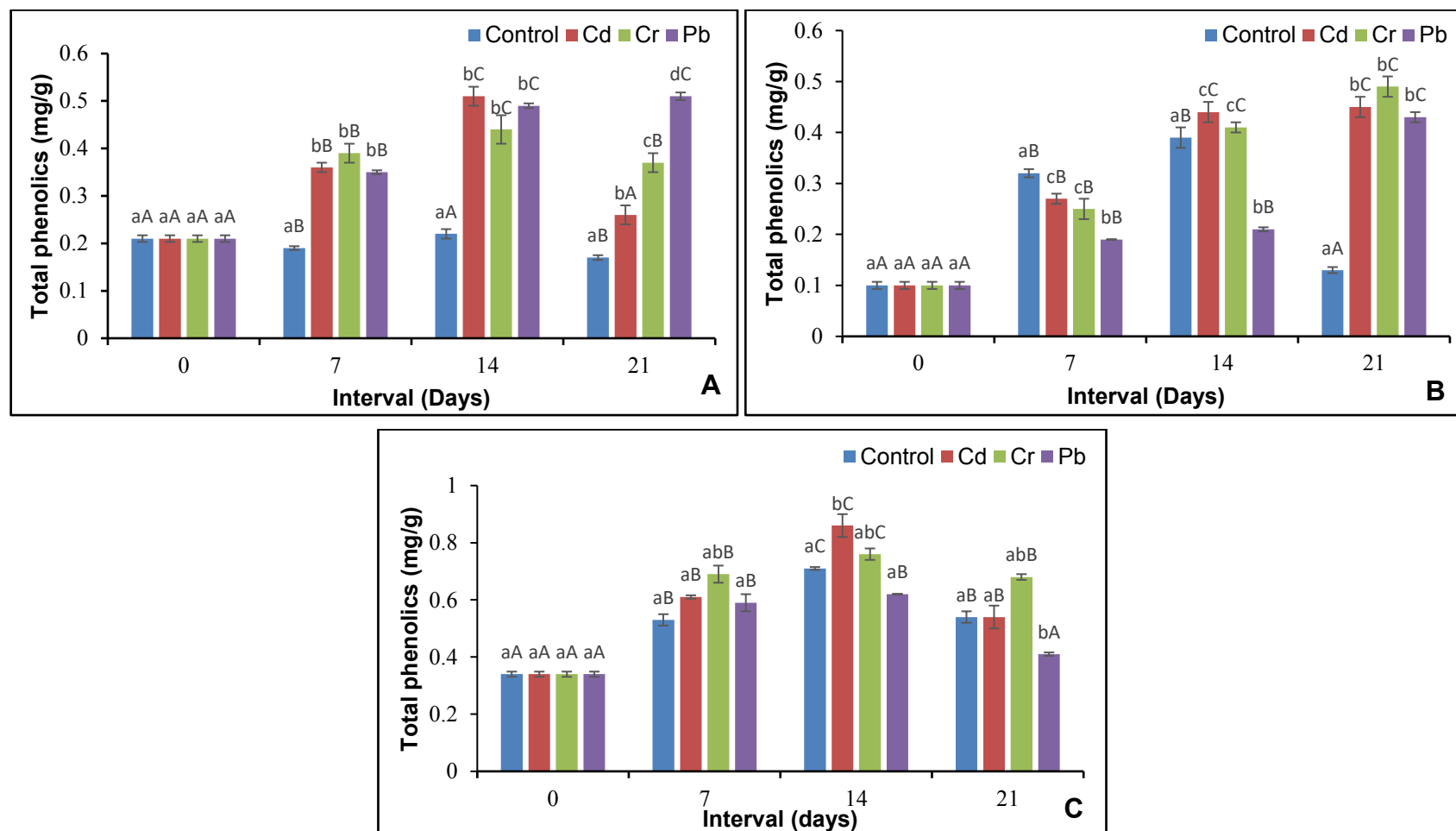


Fig 3.19. Effect of cadmium, chromium and lead on total phenolic content in *A. tenella*: A. Root, B. Stem, C. Leaf. Data represent the mean $\pm$ SE of five replicates.

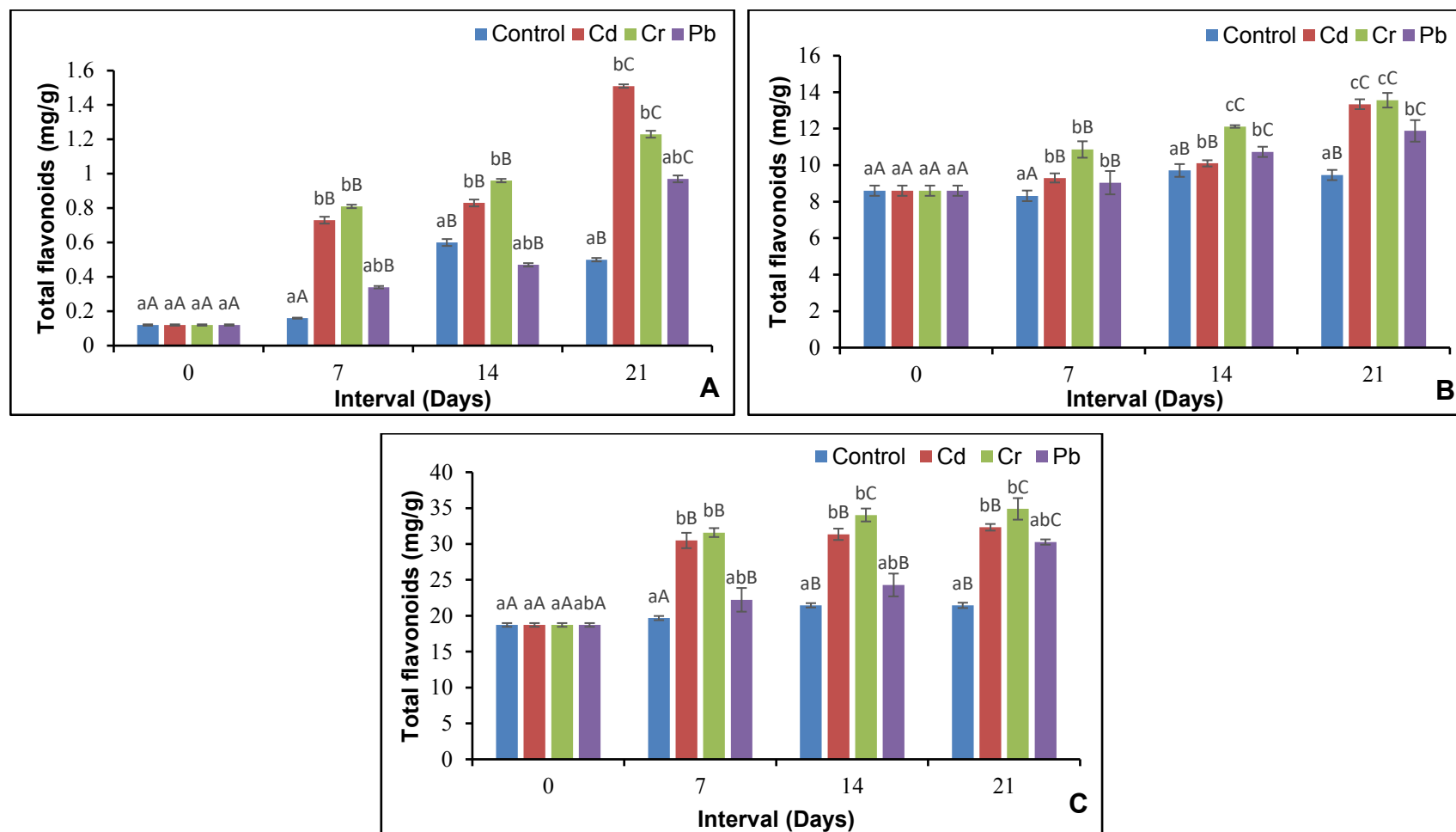


Fig 3.20. Effect of cadmium, chromium and lead on total flavonoid content in *A. tenella*: A. Root, B. Stem, C. Leaf. Data represent the mean $\pm$ SE of five replicates.

3.4.4.9. Malondialdehyde

MDA levels in control roots increased gradually from $3.43 \mu\text{mol g}^{-1}\text{FW}$ at 0d to $9.9 \mu\text{mol g}^{-1}$ at 21d, marking a 2.89-fold increase. Cd-treated roots exhibited a sharp rise in MDA levels, peaking at $44.3 \mu\text{mol g}^{-1}$ on 14d which was 12.9-fold the initial value. It was later decreased to $37.29 \mu\text{mol g}^{-1}$ at 21d. Cr exposure also caused a marked elevation, with MDA content rising to $49.29 \mu\text{mol g}^{-1}$ by 21d which is 14.37-fold the initial value and is highest among the treatments. Pb-treated roots showed moderate MDA accumulation, reaching $29.29 \mu\text{mol g}^{-1}$ at 21d (8.54-fold increase) (Fig. 3.21A).

In stems, control plants showed a gradual increase from $8.02 \mu\text{mol g}^{-1}$ to $19.01 \mu\text{mol g}^{-1}\text{FW}$ within the treatment period. Cd and Cr stress led to continuous rise in MDA levels with 3.9-fold and 4.6-fold increase, reaching $31.26 \mu\text{mol g}^{-1}$ and $36.81 \mu\text{mol g}^{-1}$ respectively on 21d. Pb-treated stems also showed a consistent increase, peaking at $30.71 \mu\text{mol g}^{-1}$ at 14d before slightly declining to $28.01 \mu\text{mol g}^{-1}$ by 21d (3.5-fold) (Fig. 3.21B).

The control leaves showed steady increase in MDA levels from $14.1 \mu\text{mol g}^{-1}$ to $25.04 \mu\text{mol g}^{-1}\text{FW}$ over 21 days. Cd stress induced a moderate increase in MDA levels, reaching $36.02 \mu\text{mol g}^{-1}$ at 14d and later dropped drastically to $14.28 \mu\text{mol g}^{-1}$ at 21d. Cr stress led to consistent surge in MDA content, peaking at $50.24 \mu\text{mol g}^{-1}$ by 21d showing a 3.56-fold increase over the initial value. Pb-treated leaves showed a 2.42-fold increase in MDA accumulation by 21d, reaching $34.16 \mu\text{mol g}^{-1}$ (Fig. 3.21C).

MDA data revealed that Cr stress induced the highest lipid peroxidation across all tissues, indicating increased oxidative damage. Cd also caused strong oxidative stress, while Pb had a moderate but consistent effect.

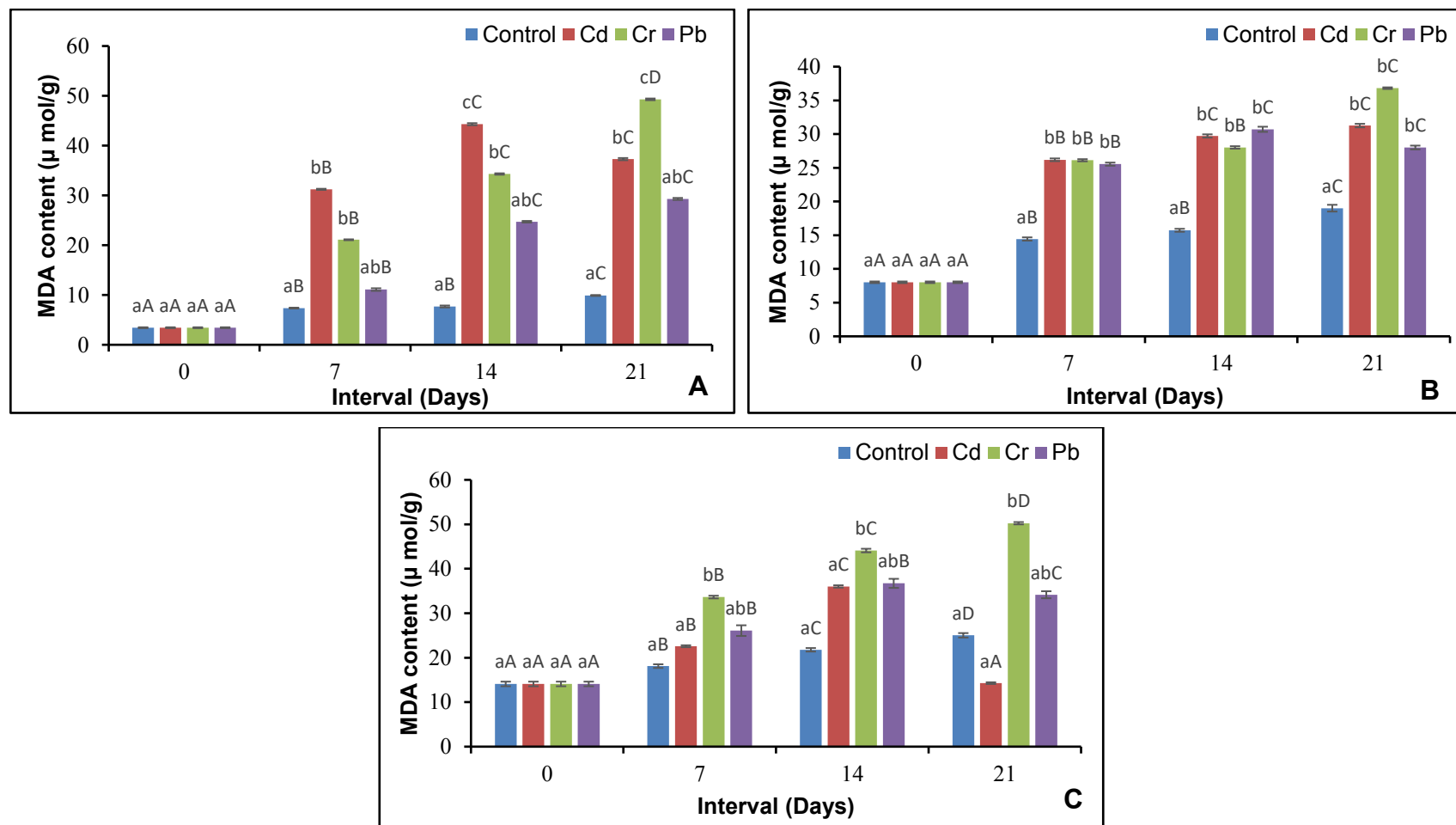


Fig 3.21. Effect of cadmium, chromium and lead on lipid peroxidation in terms of MDA content in *A. tenella*: A. Root, B. Stem, C. Leaf. Data represent the mean $\pm$ SE of five replicates.

3.4.4.10. Antioxidant enzymes

Under heavy metal stress for 21d, all antioxidant enzymes showed distinct temporal and tissue-specific responses in different parts of *A. tenella*. Pb elicited milder or delayed antioxidant responses in *A. tenella* compared to Cd and Cr.

In *A. tenella*, superoxide dismutase (SOD) activity exhibited a gradual increase over the treatment period in different plant parts under metal stress, indicating a consistent antioxidant response against superoxide radicals. SOD activity under Cd and Pb stress showed moderate increases across all tissues, with major enhancement in stems. Under Cr stress, the strongest induction of SOD activity was observed in roots followed by stems and leaves.

SOD activity showed minimal variations in control roots, ranging from 0.02 U mg⁻¹ to 0.03 U mg⁻¹ protein over the treatment period. In Cd-treated plants, SOD activity rose steadily from 0.02 U mg⁻¹ on 0d to 0.04 U mg⁻¹ protein by 21d in roots. By 21d, Cr-treated roots exhibited a gradual increase to 0.052 U mg⁻¹ protein, indicating a 2.6-fold rise over the initial activity, while a slight increase of 1.65-fold over the initial activity reaching 0.033 U mg⁻¹ protein was observed in Pb-treated plants (Fig. 3.22A).

Control stems exhibited a slight change in the enzyme activity during the treatment period, from 0.01 at 0d to 0.022 U mg⁻¹ protein at 21d. On 21d, stems showed a similar trend to root in SOD activity with a threefold increase compared to the initial level under Cd stress, reaching 0.03 U mg⁻¹ protein. Cr-treated plants showed a gradual increase from 0.01 U mg⁻¹ at 0d to 0.042 U mg⁻¹ protein, reflecting a nearly doubled SOD activity under Cr stress compared to the control at the final interval. Pb-treated plants exhibited a 1.4-fold increase from 0.01 U mg⁻¹ at 0d to 0.03 U mg⁻¹ protein (Fig. 3.22B).

Control leaves maintained low SOD activity, from 0.02 U mg⁻¹ at 0d to 0.019 U mg⁻¹ protein by 21d. In Cd-treated leaves, SOD activity was slightly reduced on 7d, but then recovered and peaked on 21d with an increase of 105% compared to control.

Cr- and Pb-treated leaves showed a 1.95-fold and 1.2-fold increase, reaching 0.039 U mg⁻¹ and 0.024 U mg⁻¹ protein respectively at 21d (Fig. 3.22C).

Catalase (CAT) activity in the roots of *A. tenella* increased drastically throughout the treatment period, while it remained relatively low and stable in control plants. Roots and leaves showed higher increase in CAT activity, while the stem showed a minimal increase under heavy metal stress. CAT activity remained low and relatively stable in control roots, declining from 0.15 U mg⁻¹ at 0d to 0.1 U mg⁻¹ at 7d and 0.09 U mg⁻¹ at 14d, and then recovering by 21d at 0.11 U mg⁻¹ protein. Under Cd stress, CAT activity increased to 1.03 U mg⁻¹ protein at 14d after an initial decline and further rose to 1.39 by 21d. Cr-treated roots exhibited a drastic increase to 1.58 U mg⁻¹ protein by 14d, reflecting more than 10-fold increase over the control at this interval. Although it was then reduced to 1.48 U mg⁻¹ protein at 21d, it remained higher comparable to control by thirteenfold. Pb-treated roots showed a progressive increase from initial value to 0.83 U mg⁻¹ protein by 21d, indicating a 6.5-fold increase over the control. This sharp increase in CAT activity in roots under heavy metal stress indicates the enhanced antioxidative defence mechanism in roots against reactive oxygen species (ROS) generated under metal stress (Fig. 3.23A).

A moderate elevation in CAT activity was observed in stems under metal treatments. with a on 21d. Plants under Cr stress recorded higher CAT activity levels at 21d (0.08 U mg⁻¹ to 0.29 U mg⁻¹ protein) in stems, which was nearly double the control value (0.13 U mg⁻¹ protein). While Cd-treated stems showed a twofold increase, Pb-treated stems recorded a 30% increase over control plants on 21d (Fig. 3.23B).

Leaves also showed a substantial increase in CAT activity under heavy metal stress. CAT activity in control plants fluctuated slightly during the treatment period, ranging from 0.2 to 0.24 U mg⁻¹ protein. The increase in CAT activity observed in Cd-, Cr-, and Pb-treated plants over the initial activity by 21d was 3.1-fold, 5.1-fold, and 1.8-fold respectively (Fig. 3.23C). This tissue-specific enhancement of CAT activity highlights its role in protecting both the primary site of metal accumulation

(roots) and photosynthetic tissues (leaves) from oxidative damage caused by heavy metal toxicity.

Heavy metal stress induced higher APX activity in roots, followed by leaves and stems. APX plays a crucial role in detoxifying H_2O_2 and protecting cellular integrity in different plant tissues under Cr stress. APX activity showed minor fluctuations in control roots during the treatment period but ended with a 29% increase over the initial value on 21d. APX activity in Cd-treated roots increased from 14.93 units/mg protein at 0d to 16.34 U mg^{-1} by 14d and peaked at 24.05 U mg^{-1} on 21d. Under Cr stress, APX activity was initially declined by 22% on 7d (from 14.93 U mg^{-1} to 11.58 U mg^{-1} protein), but subsequently increased to 17.65 U mg^{-1} at 14d and further to 22.46 U mg^{-1} protein by 21d. This marks a 1.35-fold increase over the control at the final interval and suggests a late-phase activation of ROS-scavenging mechanism under prolonged Cr exposure. Pb-treated roots also showed an initial decline to 9.25 U mg^{-1} at 7d, followed by a drastic increase to 15.58 U mg^{-1} at 14d, and peaked at 25.6 U mg^{-1} protein by 21d. This marks a 54% increase in APX activity under Pb stress over control at the final interval (Fig. 3.24A). The enhancement in APX activity in the roots of *A. tenella* can be observed as an intensified detoxification of hydrogen peroxide (H_2O_2) in the roots, which is the primary site of Pb uptake and initial oxidative stress.

Both the control stems and leaves exhibited fluctuations in the APX activity throughout the treatment period. APX activity in control stems declined to 1.03 U mg^{-1} protein at 7d, followed by an increase at 14d to 1.84 U mg^{-1} protein, and then declined to 1.67 U mg^{-1} protein by 21d. Cd-treated stems showed a nearly sixfold increase over the initial activity by 21d. Both Cr- and Pb-treated stems showed a consistent and pronounced increase from 1.23 U mg^{-1} at 0d to ~5 U mg^{-1} protein at 21d, marking more than a threefold increase over the control (Fig. 3.24B).

Control leaves showed an increase from 6.03 U mg^{-1} at 0d to 9.01 U mg^{-1} protein at 21d, after declining in the initial weeks. Under Cd and Cr stress, a significant increase in the APX activity of 24.75% and 34.5% respectively was noted in leaves by 21d. This variation suggests a delayed yet substantial activation of the APX system in leaves in response to these metal-induced oxidative stress. APX activity

in Pb-treated plants was comparable to control in all intervals of treatment (Fig. 3.24C).

GPX activity in control roots showed a slight gradual increase from 2.82 U mg⁻¹ at 0d to 3.29 U mg⁻¹ protein on 21d. Under Cd stress, GPX activity in the roots declined slightly during the early phase (about 8% on 7d), but later showed a gradual increase by 32% on 21d. Cr-treated roots showed a consistent rise reaching 4.12 U mg⁻¹ at 21d, whereas, a marked reduction reaching 1.6 U mg⁻¹ protein at 21d, reflecting a 51% reduction from the control value was observed in Pb-treated roots (Fig. 3.25A).

Control stems showed a modest increase in the GPX activity over time, from 1.08 U mg⁻¹ at 0d to 1.7 U mg⁻¹ protein by 21d. All metal-treated stems exhibited a similar trend comparable to controls during the initial weeks. However, the GPX activity of stem under Cd and Cr treatments peaked on 21d with 2.68 U mg⁻¹ protein and 2.45 U mg⁻¹ protein respectively, indicating a 1.6-fold and 1.4-fold increase over control. Pb-treated stems also showed a modest increase in the GPX activity, though the activity remained consistently lower than controls throughout the period. GPX activity in stems under Pb stress reached 1.54 U mg⁻¹ protein on 21d, reflecting a decrease of 68.23% over the control (Fig. 3.25B).

Control leaves showed low and stable activity, from 0.59 U mg⁻¹ at 0d to 0.98 U mg⁻¹ protein at 21d. The enhancement of GPX activity was more pronounced in the Cd- and Cr-treated leaves. There was a sharp and gradual increase from 2.11 U mg⁻¹ on 7d to 3.51 U mg⁻¹ protein by 21d under Cd stress. This 66% increase over initial activity highlights the role of GPX in protecting photosynthetic tissues from Cd-induced ROS. Cr-treated leaves also showed significant enhancement in GPX activity reaching 4.26 U mg⁻¹ protein at 14d. It was later slightly reduced to 3.44 U mg⁻¹ protein by 21d. GPX activity in leaves, however, was significantly enhanced under Pb treatment. Pb-treated leaves also exhibited a increase, reaching 2.94 U mg⁻¹ protein at 21d, indicating a fivefold increase over the initial value. This represents 200% increase compared to control at the final interval, compensating for elevated H₂O₂ levels (Fig. 3.25C).

These enzyme responses highlight efficient antioxidant defence of *A. tenella* under heavy metal stress.

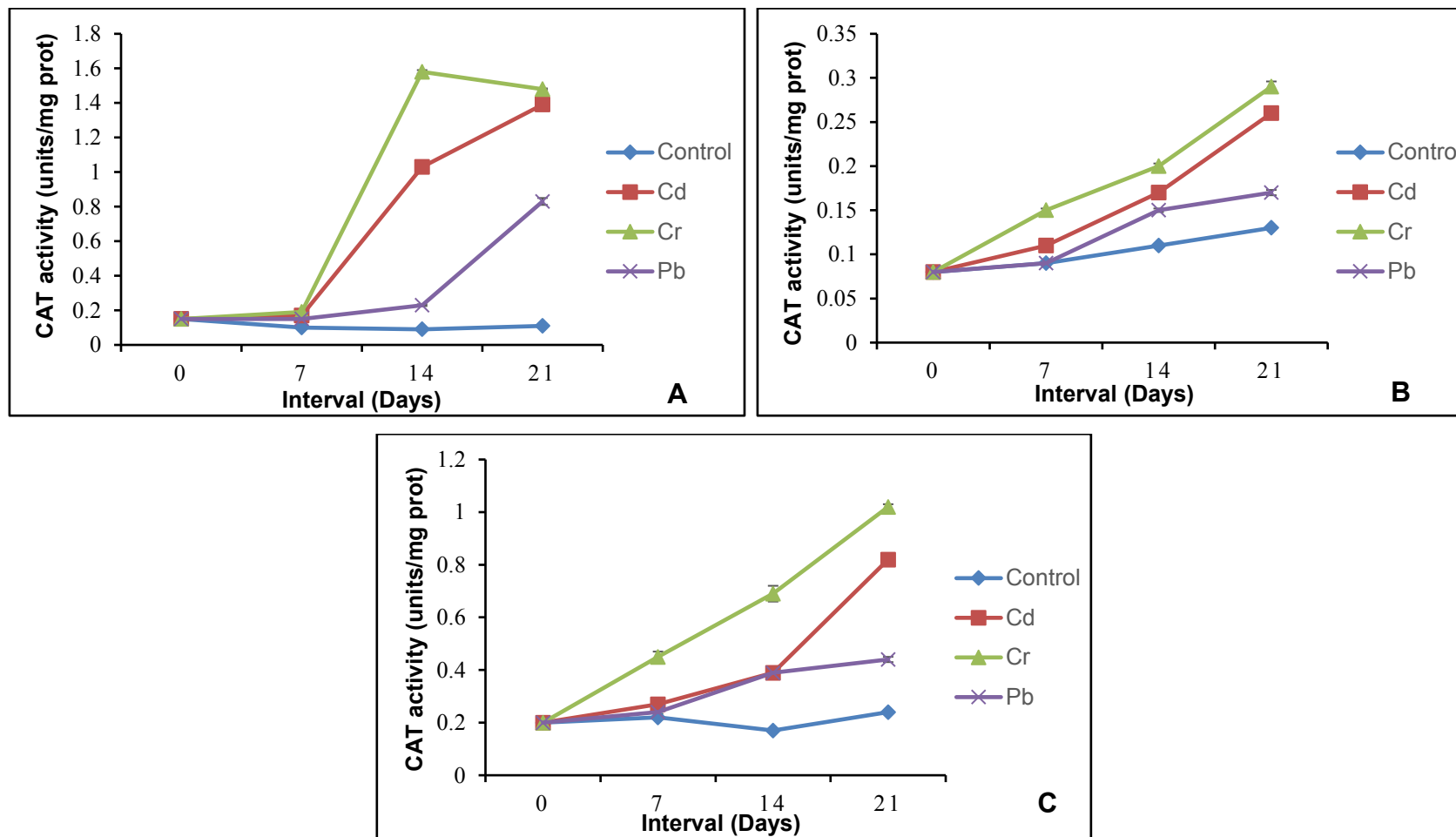


Fig 3.22. Effect of cadmium, chromium and lead on catalase activity in *A. tenella*: A. Root, B. Stem, C. Leaf. Data represent the mean $\pm$ SE of five replicates.

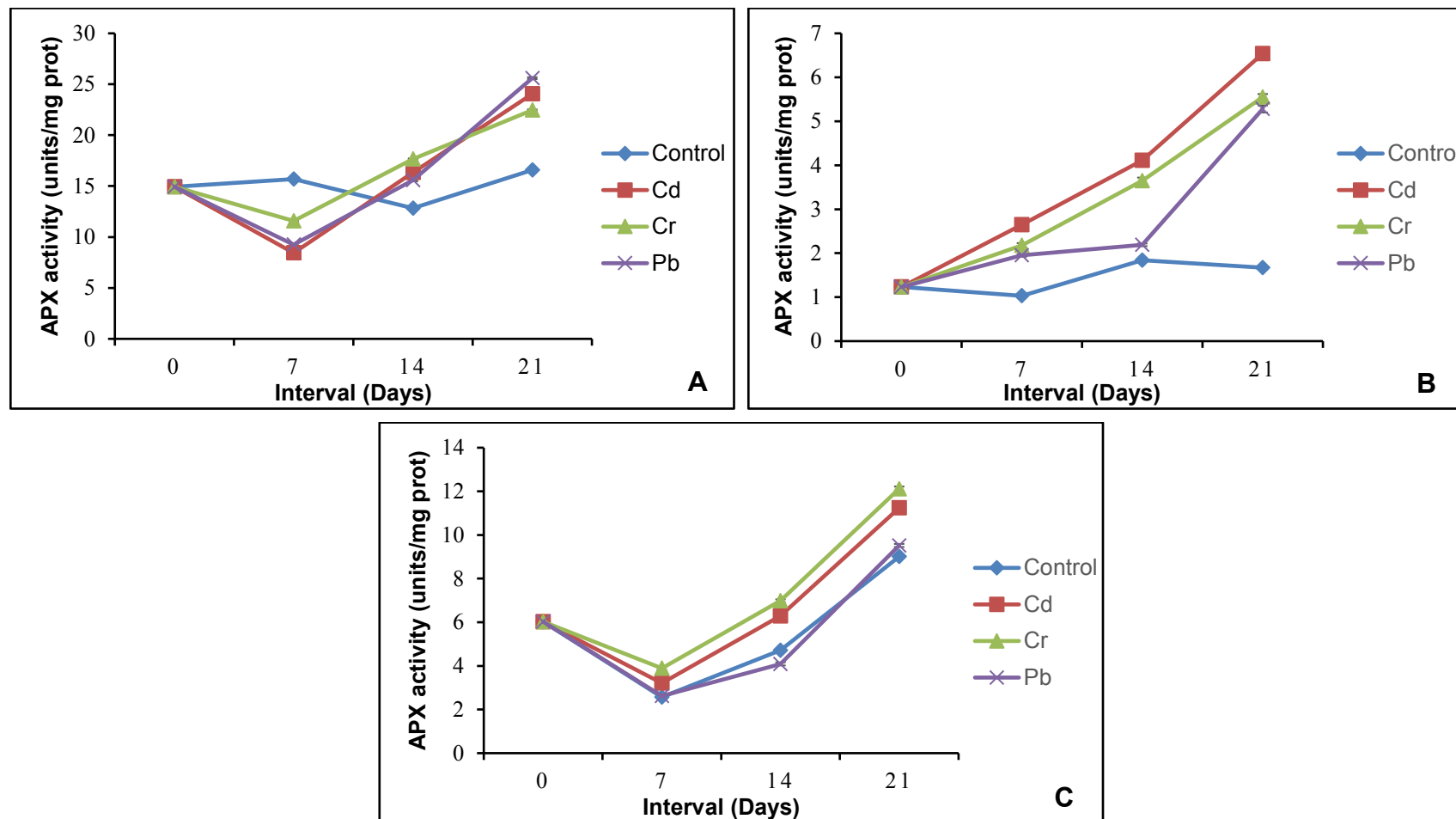


Fig 3.23. Effect of cadmium, chromium and lead on ascorbate peroxidase activity in *A. tenella*: A. Root, B. Stem, C. Leaf. Data represent the mean $\pm$ SE of five replicates.

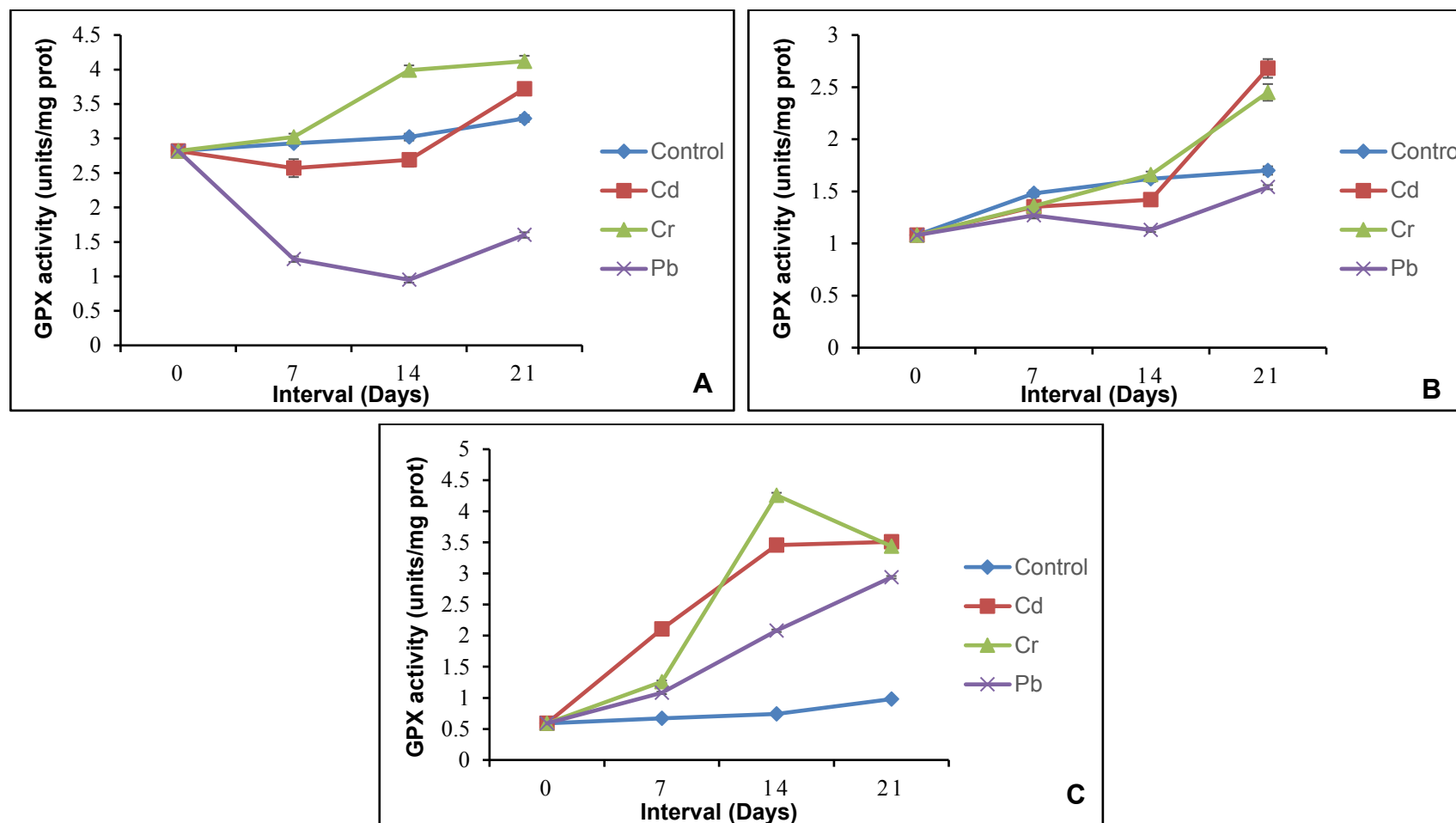


Fig 3.24. Effect of cadmium, chromium and lead on guaiacol peroxidase activity in *A. tenella*: A. Root, B. Stem, C. Leaf. Data represent the mean $\pm$ SE of five replicates.

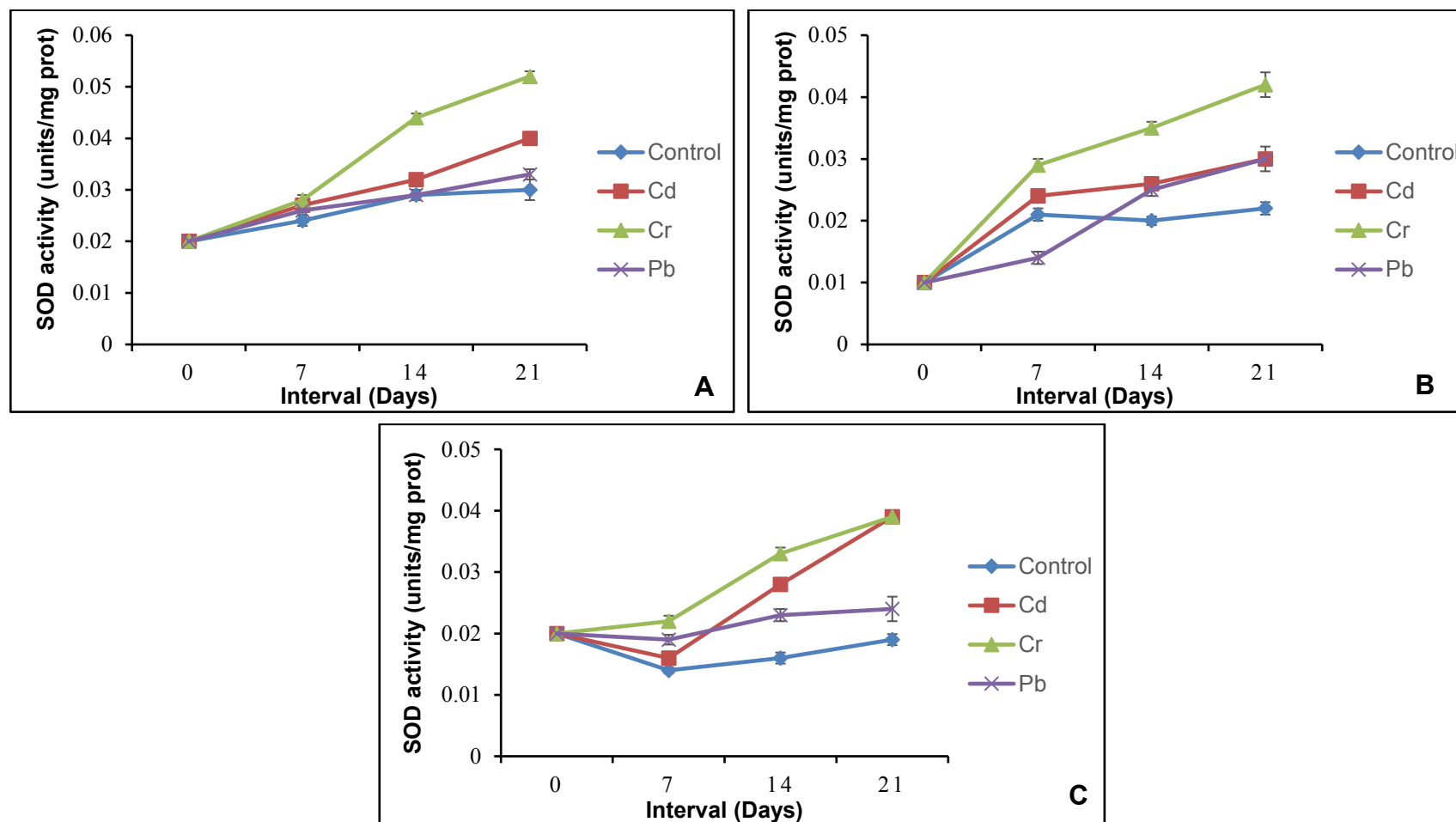


Fig 3.25. Effect of cadmium, chromium and lead on superoxide dismutase activity in *A. tenella*: A. Root, B. Stem, C. Leaf. Data represent the mean $\pm$ SE of five replicates.

3.4.5. Fourier transform infrared (FTIR) analysis

The functional groups of biomolecules (carboxyl, phosphate, amide, and hydroxide) that interact with transition metals in biological samples such as phenols, aldehydes, and carbohydrates, can be identified by analysing the infrared light adsorption of the molecules. Thus, FTIR spectroscopy serves as an effective tool to study metal cation interactions in biological samples. In this study, IR spectra of roots, stems, and leaves from both control and heavy metal-treated samples were analysed to detect changes in functional group composition under metal stress. Notable peak shifts were observed in treated samples across different IR regions, indicating alterations in the chemical composition and structural properties of biomolecules (Fig. 3.26 A-C). These spectral changes were interpreted with reference to control samples, and functional group assignments were made based on established literature (Peng *et al.*, 2020).

After 21d of growth, control root samples had absorption peaks at 3412, 2925, 2684, 2357, 2145, 1641, 1360, 1060, and 615 cm^{-1} . The absorption peaks of control stem samples were at 3419, 2922, 2354, 2132, 1640, 1382, 1054, and 610 cm^{-1} and that of control leaf samples had absorption peaks at 3423, 2925, 1627, 1384, 1316, 1241, 1060, 780, and 517 cm^{-1} .

Cd-treated root samples had absorption peaks at 3419, 2922, 2356, 2125, 1639, 1383, 1052, and 606 cm^{-1} . The absorption peaks of Cd-treated stem samples were at 3423, 2922, 2687, 2354, 2134, 1635, 1382, 1057, and 662 cm^{-1} and that of Cd-treated leaf samples were at 3415, 2922, 1638, 1384, 1318, 1051, 780, and 517 cm^{-1} . The notable changes were observed in the 3200–3500 cm^{-1} region, corresponding to N-H groups of primary amines (protein) and O-H groups (alcohols, phenols, carboxylic acids) of lignin, cellulose, saponin, and tannin (Bora and Sarma, 2021). Alterations were also noted in the 2600–2000 cm^{-1} range, characteristic of symmetric stretching vibrations of groups like O=C=O and N=C=N, as well as in the 1600–1670 cm^{-1} range, representing symmetric stretching of C=C. The 1400–1000 cm^{-1} region, characterized by groups S=O, C-O, C-N, and C-F, also exhibited notable alterations. Additionally, changes were observed in the 900–1100 cm^{-1} region, characterized by

symmetric stretching of $>P=O$ of phosphodiesteres, and in the $400\text{--}800\text{ cm}^{-1}$ region, attributed to $-C-OH$ stretching vibrations and $C-S$ stretching of disulfide. These shifts collectively indicate significant modifications in the molecular structure and composition of proteins, nucleic acids, phospholipids, and other cellular components in response to Cd stress.

Cr-treated root samples had absorption peaks at 3418, 2924, 2356, 2120, 1638, 1381, 1054, and 603 cm^{-1} . The absorption peaks of Cr-treated stem samples were at 3421, 2922, 2354, 2090, 1638, 1382, 1055, and 662 cm^{-1} . Cr-treated leaf samples had absorption peaks at 2980, 1641, 1384, 1250, 1071, and 953 cm^{-1} . No significant changes were observed in the absorption peaks of control and Cr-treated samples in case of root and stem tissues which means that the interaction of heavy metal ions with all functional groups was less. In case of leaf samples, the absorption peaks have some variations which shows that the dry biomass had different functional groups available for binding of heavy metal ions. No absorption peaks were observed in the $3600\text{--}3200\text{ cm}^{-1}$ range in the Cr treated sample which characterize O-H and N-H stretch, but a peak at 3423 cm^{-1} was recorded in control sample. Also, there were absorption peaks below 900 cm^{-1} in the control samples which were absent in the Cr treated leaf sample.

After 21d of growth, Pb-treated root samples had absorption peaks at 3415, 2922, 2354, 2128, 1637, 1383, 1057, and 606 cm^{-1} , whereas the absorption peaks of Pb-treated stem samples were at 3421, 2921, 2354, 2119, 1640, 1382, 1057, and 662 cm^{-1} . Pb-treated leaf samples had absorption peaks at 3423, 2921, 1640, 1384, 1250, and 1051 cm^{-1} . In Pb-treated samples, the presence of a peak in the $3200\text{--}3600\text{ cm}^{-1}$ region is characteristic of a symmetrical O-H stretch caused by hydrogen bonds within the compound. Additionally, absorption in this region is associated with hydrogen atoms bound to carbon, oxygen, and nitrogen (C-H, O-H, and NH) (Deguchi, 2006). The association between metals and plant tissues may be related to these chemical bonds, as the intensity of the peaks in this region increases with metal treatment.

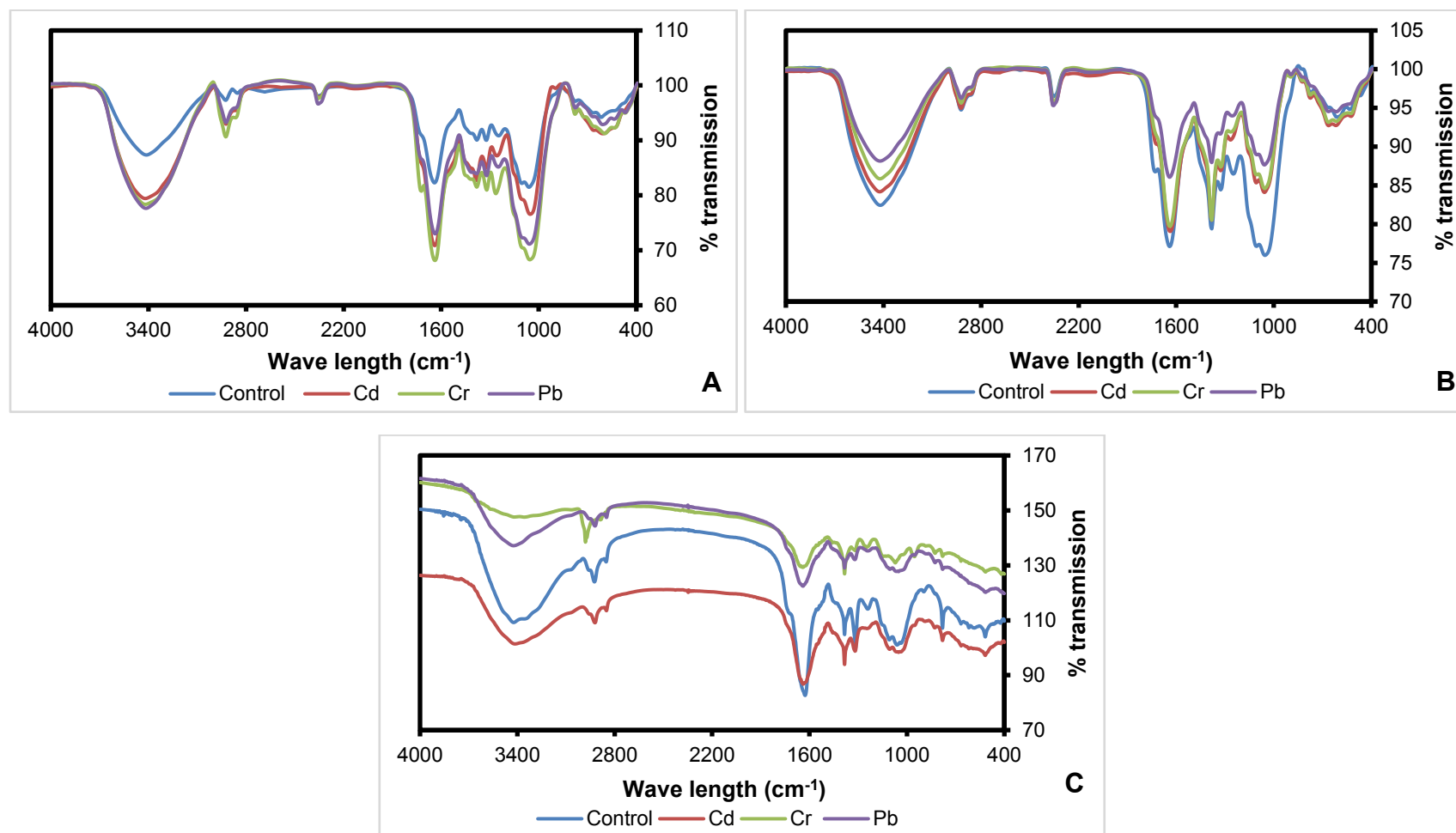


Fig 3.26. FTIR spectra of *A. tenella* subjected to metal treatments compared to control: A. Root, B. Stem, C. Leaf

The regions around 2600-2950 cm^{-1} are attributed to C-H stretching in chlorophyll, glycerolipids, and wax hydrocarbons. Bands at 1641.128 and 1637.528 cm^{-1} and 1627.18 and 1640.47 cm^{-1} correspond to specific amide groups of proteins due to C=O stretching. Additionally, the stretching of cellulose and lignin in leaves is observed with the disappearance of peaks at 1316.42 cm^{-1} and 1241.11 cm^{-1} , which are only present in control spectra. The regions from 1200-900 cm^{-1} signify overlapping C-C, C-O, and C-O-P stretches (Wolkers *et al.*, 2004), occurring mainly in cellular polysaccharides. The 700-400 cm^{-1} region also characterizes -OH (Barud *et al.*, 2013). Compared to the control, Pb-treated *A. tenella* biomass shows a significant increase or decrease in peak intensity, with certain peak bands completely disappearing in the treatment. This can be attributed to an increase in Pb content in Pb-treated *A. tenella*, which alters infrared spectra absorbance.

3.4.6. Heavy metal biodistribution in plant tissues

A. tenella plantlets were exposed to 170 μM of Cd, 240 μM of Cr (VI), 680 μM of Pb for a period of 21d. The results showed that the roots had a significantly higher concentration of metals compared to the aerial parts of plant, indicating that majority of the metals taken up by the plantlets was retained in the roots and only a small portion was transported to the stem and leaves. The order of metal concentrations was root > stem > leaf.

On 21d, the roots of treated *A. tenella* had a Cd content of 317.25 mgkg^{-1} DW, while the stem and leaves of plants had Cd concentrations of 87.462 mgkg^{-1} DW and 72.825 mgkg^{-1} DW respectively (Table 3.9). The plant showed a total Cd accumulation ($\text{Cd}_{\text{root}} + \text{Cd}_{\text{shoot}}$) of 5925.608 mgkg^{-1} DW on 21d, with Cd accumulation rate in the plant and distribution proportion of Cd in root as 282 and 66% respectively. The total Cr concentration in the roots of the plantlets was 179.625 mg/kg DW on 21d (Table 3.9). The accumulation of Cr in the stem and the leaves was less as compared to the roots. The total Cr concentration in the stem and leaves of the plantlets was 87.075 mg/kg DW and 98.625 mg/kg DW, respectively on 21 d. The plant showed a total Cr accumulation of 4564.869 mgkg^{-1} DW on 21d. Cr accumulation rate in the plant and distribution proportion of Cr in root were 217

and 44% respectively. The Pb content in the roots of the treated *A. tenella* was 2682.5 mg/kg DW on 21d, while the stem and leaves had Pb concentrations of respectively 324.75 mg/kg DW and 212.375 mg/kg DW (Table 3.9). The total Pb accumulation (Pbroot + Pbshoot) in the plants was 46,866.921 mg/kg DW on 21d. While the Pb accumulation rate in the plant was 2232.7, distribution proportion of Pb in roots was 83%.

The BCF and BAC were used to estimate the metal concentration in the roots and shoots (including the stem and leaves) respectively against the metal concentration in the treatment medium. The BCF value was given by the ratio of the concentration of metal in roots of treated plant to that of medium and was found to be 8.39 and 16.23 for *A. tenella* under Cd and Cr treatment respectively. The BAC value measures the proportion of metal concentration in shoot (stem and leaves) to that of medium. The BAC value of Cd and Cr-treated *A. tenella* was equally high, at 16.6 and 15.7 respectively. For *A. tenella* under Pb treatment, the BCF and BAC values were determined to be 19.04 and 3.81, respectively. Translocation factor (TF) measures the concentration of metal translocated from the root to the aboveground part of the plant. TF value was calculated to be 0.505 and 0.2 for Cr and Pb-treated *A. tenella*, which indicates that *A. tenella* preferentially accumulates these metals in the roots, making it suitable for the phytostabilization of Cd and Pb. For Cr-treated *A. tenella*, TF was found to be 1.03 (TF >1) which can make it ideal for phytoextraction of Cr.

Table 3.9. Metal concentration and accumulation in different parts of metal-treated *A. tenella* on 21d.

Sample	Cd concentration [Cd]	Cd accumulation [Cd]*DW	Cr concentration [Cr]	Cr accumulation [Cr]*DW	Pb concentration [Pb]	Pb accumulation [Pb]*DW
	(mgkg ⁻¹)	(mgkg ⁻¹ DW)	(mgkg ⁻¹)	(mgkg ⁻¹ DW)	(mgkg ⁻¹)	(mgkg ⁻¹ DW)
Leaf	72.825	1039.941	98.625	1505.0175	212.375	3667.716
Stem	87.462	1532.33424	87.075	1373.17275	324.75	6261.18
Root	317.25	3353.3325	179.625	1686.67875	2682.5	36938.025
Total	477.537	5925.60774	365.325	4564.869	3219.625	46866.921

3.5. Discussion

3.5.1. Plant growth

The interactions of heavy metals with enzymes and biochemical reactions in plant tissues obstruct plant metabolism, adversely affecting overall plant development and growth. The onset of HM toxicity in plants is often marked by visible morphological alterations such as leaf chlorosis, root and shoot growth retardation, wilting, low biomass accumulation, and necrosis. In *A. tenella*, exposure to HM stress negatively impacted plant growth, leading to reduced root and shoot length, leaf area, and biomass. Reduction in these parameters is correlated with the increase in days of exposure to these metals. Roots are the primary sites for metal toxicity since they are directly associated with heavy metal uptake. As a tolerance mechanism in plants, stress-induced root length reduction reduces the region of exposure to metal stress (Miras-Moreno *et al.*, 2014).

Inhibition of root length elongation and biomass is an early and prominent effect of Cd toxicity, stemming from decreased mitotic activity in root meristems caused by chromosome aberrations and depolymerization of microtubules in the cell cytoskeleton under Cd stress (Subašić *et al.*, 2022; Shanying *et al.*, 2017). The toxicity of Cd to plant structure and photosynthetic organs likely contributed to the decrease in net growth of plant height and biomass (Zhao *et al.*, 2021). The retardation in plant growth may also result from limited uptake of essential minerals and reduction in the production of photo assimilates (Wang *et al.*, 2008). Reduction in growth parameters due to Cd stress have been reported in several plants like *Eruca sativa*, *Erigeron annuus*, and *Vicia faba* (Waheed *et al.*, 2022; Zhang *et al.*, 2021; Piršelová *et al.*, 2021).

Cr (VI)-mediated root growth inhibition may result from a reduction in cell size and an inhibition of cell division in the elongated region (Peralta *et al.*, 2001) or the chromosome distortion in root apex cells, or the stress ethylene production (Shinwari *et al.*, 2015; Shahid *et al.*, 2017). Plants take up Cr through roots and it gets transported to shoot, which affects the growth of stem and leaves. The Cr stress-

induced decrease in shoot length may be due to ultrastructural damage in leaf mesophyll cells, which impairs photosynthesis and ultimately results in decreased shoot development (Singh *et al.*, 2021). The reduction in root and shoot length and leaf area was supported by the studies on *Citrus x aurantium* L. (Shiyab, 2019), *Ipomoea ternata* Jacq. (Rhaman *et al.*, 2020), *Vigna radiata* (Singh *et al.*, 2021), and *Oryza sativa* (Chen *et al.*, 2017). The phytomass get reduced as the Cr gets translocated from root to above aerial parts of the plant in *A. tenella*. This is in correlation with the reports of Jayed *et al.* (2021) in *Solanum lycoperscium*, Kamran *et al.* (2021) in *Brassica rapa*, Basit *et al.* (2022) in *Glycine max* and Singh *et al.* (2021) in *V. radiata*.

Roots respond to Pb exposure by altering their length, volume, and diameter, as well as by producing or inhibiting lateral roots. The rapid inhibition of root growth is a primary effect of Pb toxicity in plants, which may result from inhibited cell division and/or cell elongation in root tips (Fahr *et al.*, 2013). This inhibition could be due to metal-induced chromosomal abnormalities, altered cell wall flexibility, decreased glutathione pool, suppression of microtubule growth, disrupted DNA synthesis, and an extended mitotic cycle (Osman *et al.*, 2023). Abnormalities in cell mitosis also affect the growth of stem under Pb toxicity (Sharma and Dubey, 2005). Pb toxicity typically decreases plant biomass by limiting the nutrient uptake, nitrogen metabolism, and photosynthesis inhibition (Shehzad *et al.*, 2023), as observed in *A. tenella*. Pb induced reduction in plant growth parameters under Pb toxicity have been reported in various plants, including *Leucaena leucocephala* (Alkhatib *et al.*, 2019), *Albizia lebbeck* (Naveed *et al.*, 2023), *Brassica* spp. (Shehzad *et al.*, 2023), and rice (Khan *et al.*, 2021).

According to Karasov *et al.* (2017), a plant's defence mechanisms cause a reduction in growth and reproduction under stress conditions. This suggests that the decreased growth rate and biomass in *A. tenella* may be a strategy to cope with the deleterious effects of HMs.

Tolerance indexes based on root length are regarded as a good metric for determining how resilient plants are to heavy metal stress (Majeed *et al.*, 2019). The

low TI value found in *A. tenella* after Cd and Cr treatment suggests that the high concentration of these metals interferes with cell division and severely hinders root growth. The higher TI shown by Pb-treated plants indicates that *A. tenella* can withstand Pb stress more effectively, maintaining its growth relatively to a control plant.

3.5.2. Anatomical modifications

Significant anatomical modifications were observed in various parts of *A. tenella* in response to metal stress. SEM images revealed distortion of cell structures, thickening of cell walls, and occlusions in vascular elements and parenchyma cells as major changes due to heavy metal uptake and accumulation. Scanning micrograph of control root and shoot showed the distinct features of epidermis, cortex, vascular region and pith, but with metal treatment these tissues got distorted. Roots and stems exhibited more damage compared to leaves under HM stress, showing severe deformation of cell structures and the presence of deposits within tissues, consistent with previous studies (Bora and Sarma, 2021; Liza *et al.*, 2020; Wakeel *et al.*, 2020; Alkhatib *et al.*, 2019; Alves *et al.*, 2014).

Depositions were clearly observed in root tissues of Cd-treated *A. tenella*, indicating predominant Cd retention in the roots. This is like the results in *A. ilicifolius*, suggesting the complexation of Cd with phytochelatin in vacuoles to impede vertical Cd transport via xylem (Shackira and Puthur, 2019). Limited Cd translocation to aerial parts is attributed to apoplastic Cd fractions in roots and binding to cell walls, serving as a defence mechanism against Cd toxicity. According to previous reports, various polysaccharides like pectin, cellulose, and proteins of the cell wall are responsible for Cd binding to negatively charged sites (Chandrasekhar and Ray, 2019; Bora and Sarma, 2021). The findings from FTIR analysis further corroborate the binding of Cd to polysaccharides and proteins, evidenced by the shifting of their respective peak positions in the Cd-treated biomass of *A. tenella*. The thickening and deformation of cell walls act as barriers against HM transport by resisting radial water flow (Pandey *et al.*, 2022). Changes in xylem structure, including depositions and alterations in vessel characteristics,

further hinder HM entry and transport to the plant shoot (Yadav *et al.*, 2021). Cd-induced anatomical changes, such as reduced xylem tracheid diameter, can be attributed to lignin deposition in the cell wall, consistent with previous literature (Bora and Sarma, 2021). Up-regulation of lignin biosynthesis and lignifying enzymes such as peroxidases under Cd stress suggests a defence response and mechanical adaptation (Rui *et al.*, 2016; Liu *et al.*, 2018). Anatomical changes in roots under Cd stress reduce water and mineral uptake, inhibiting plant growth (Pérez Chaca *et al.*, 2014); hence validating the results of the morphological and EDAX studies. In the stem, xylem elements and surrounding tissues are primarily affected sites for HM entry via the vascular system (Pandey *et al.*, 2022). The reduction in stem diameter, number and size of xylem vessels, increased cell wall thickness, number of trichomes and sclerenchyma above the phloem have been reported as an act of defence against Cd stress in plants by several authors (Yadav *et al.*, 2021; Liza *et al.*, 2020; Shackira and Puthur, 2019). Limited metal translocation to leaves is a defensive strategy to safeguard the photosynthetic system, with even small doses of HM causing severe anatomical changes, such as reduced cell and vessel size, affecting pigment synthesis and stomatal parameters (Pandey *et al.*, 2022). Most stomata in Cd-treated *A. tenella* leaves appeared closed, a common response to metal exposure (Batool *et al.*, 2015). Stomatal density, size, and partial/full closure decrease under Cd stress. Increased Ca^{2+} levels in roots or abscisic acid accumulation in guard cells induce leaf turgor reduction and stomatal closure, limiting gas exchange (Rucinska-Sobkowiak, 2016; Sha *et al.*, 2019). Some stomata were widely opened with thick guard cells and visible depositions, possibly indicating Cd transpiration, making the plant suitable for phytovolatilization (Yan *et al.*, 2020).

Chromium (VI) on passing through endodermis via symplast probably get reduced to Cr (V) which gets retained in the root cortex cells and this may be the reason for distortions in the root. Continued exposure to Cr can lead to the translocation of Cr from root to stem. At higher concentrations of Cr, reactive oxygen species (ROS) production is enhanced that cause oxidative damage at cellular level and this may be the reason for distortions in anatomy of stem (Shanker *et al.*, 2005). Shanker *et al.*

(2004) has also mentioned that the damage of root and stem tissues can be the result of cell malfunctioning induced by Cr accumulation in roots, by the displacement of Ca^{2+} ions from the binding sites in cell walls and plasma membranes. The sequestration of Cr in the root vacuoles and poor translocation from root to shoots thus alters the structure of the root (Shanker *et al.*, 2004) and in *A. tenella*, it can be correlated with the reduced root growth. Scanning electron micrographs showed the presence of some aggregates on the cell wall of xylem elements of the Cr treated leaves. Rucinska-Sobkowiak (2016) reported that apoplast resistance to water flow is increased by cell wall thickening caused by metal deposits and/or incrusting substances in the cell walls. This will in turn eventually reduces the transportation of water and soluble mineral nutrients from the root to the shoot, and this observation can be corroborated with the decreased macro and micro-elements content detected in shoot than roots in the SEM-EDAX data. Fully opened stomata were observed on the leaf surface of control plants, while the stomata in Cr treated plants were partially opened. Moreover, in *Brassica juncea* (L.) Czern. treated with Cr, sunken and poorly defined elliptical stomata were seen (Han *et al.*, 2004). This may be due to rapid induction of stomata closure by abscisic acid (ABA), a signalling molecule activated under water stress to reduce the water loss from leaves (Rucinska-Sobkowiak, 2016).

Pb deposition was notably observed in the root tissues of Pb-treated *A. tenella*, indicating predominant Pb retention in the roots. The dark depositions observed in the xylem walls and cortical parenchyma of the roots are the Pb ions bound to the cell wall. This acts as an anatomical defence mechanism that reduces the cytosolic concentration of heavy metals, helps maintain hydraulic safety, and prevents water loss (Enstone *et al.*, 2003). In Pb-treated *A. tenella* roots, structural anomalies such as withered cells, nicks, and irregularities in root surface cells were observed, which can disrupt cell wall and membrane integrity. This finding aligns with the results of Kaur *et al.* (2013) and Liu *et al.* (2009) regarding the effects of Pb on *Triticum aestivum* and *Allium sativum*. The distortion of cell shape in Pb-treated plants is likely due to the disorientation of cellulose micro fibrils, caused by Pb^{2+} binding to membranes, which separates granular material from the plasma membrane and

inhibits polysaccharide chain growth (Ahmad *et al.*, 2005). This finding supports the shift in FTIR peaks corresponding to polysaccharides bound with Pb in the Pb-treated biomass of *A. tenella*. Kaur *et al.* (2013) also reported that Pb stress activates certain wall-degrading enzymes in roots, leading to distensions and lesions in the cell wall. In *L. leucocephala*, exposure to high Pb concentrations caused thickened cell walls and plasma membrane invaginations, indicating severe cellular stress and attempts to mitigate Pb toxicity through physical barriers (Alkhatib *et al.*, 2019). The thickening of the root exodermis and endodermis serves as a barrier to Pb transport and a site for metal accumulation, as observed in *Oxycaryum cubense* (Alves *et al.*, 2014). It can also be an adaptive strategy in plants to limit metal translocation, as seen in *Brachiaria decumbens* (Gomes *et al.*, 2011). Thickened cell walls increase the retention area for toxic metals, minimizing their harmful effects and reducing their movement into the plant shoot (Krzesłowska, 2011; Gomes *et al.*, 2011). Anatomical alterations in roots under Pb stress impair water and mineral intake, which decreases the plant growth (Alves *et al.*, 2014), supporting our morphological and EDAX results. Pb is reported to cause an increase in stem diameter and thickness, number and size of xylem vessels, and increased cell wall thickness (Petrovic *et al.*, 2021). Even small doses of heavy metals cause severe anatomical changes in leaves, such as reduced cell and vessel size, affecting stomatal parameters and pigment synthesis. Therefore, limiting metal translocation to leaves acts as a defence mechanism to protect the plant's photosynthetic system (Pandey *et al.*, 2022). The electron-dense depositions seen in the cell wall, intercellular spaces, and the vacuoles of Pb-treated leaf cells was also observed in *O. cubense* and *Lemna minor* (Alves *et al.*, 2014; Samardakiewicz and Wozny, 2000). These depositions likely result from increased apoplastic transport of Pb and serve as a key resistance mechanism against toxic effects of metal, protecting cell metabolism and enhancing plant tolerance (Piechalak *et al.*, 2002; Seregin and Ivanov, 2001). In Pb-treated *A. tenella* leaves, some stomata were widely open like those in the control, while others were completely closed. Pb induced stomatal closure has been reported in several plants including soybean, tobacco, wheat and *L. leucocephala* (Alkhatib *et al.*, 2019; Alkhatib *et al.*, 2012; Kanwal *et al.*, 2020; Weryszko-Chmielewska and Chwil, 2005).

Stomatal closure under metal stress may result from increased levels of abscisic acid (ABA), which causes water loss in guard cells, or from ion imbalance by affecting calcium channels in guard cells. Heavy metals may also directly reach guard cells via root uptake, leading to stomatal closure (Guo *et al.*, 2023). Heavy metal exposure generally reduces stomatal conductance (opening), which affects plant transpiration. However, some studies report increased stomatal conductance due to low internal CO₂ levels prompting plants to open stomata for more CO₂ uptake or potassium ion leakage impairing stomatal closure regulation (Xiong *et al.*, 2019). This supports the presence of opened stomata in *A. tenella* under Pb stress.

3.5.3. Element analysis in tissues

Mineral nutrients are essential for plant growth and development, but they get reallocated to tolerate stress (including HM stress) in plants under adverse environmental conditions (Jalloh *et al.*, 2009).

In soil, cadmium typically exists as Cd²⁺ ions and Cd-chelates. Its uptake in plants primarily occurs through interaction with absorption sites, often associated with other nutrient minerals such as Ca²⁺ channels found in the guard cells of plasma membranes (Haider *et al.*, 2021). Utilizing the apoplastic pathway, regulated by membrane potential, Cd enters the roots and is subsequently translocated either in its ionic form through transporters or via the ascent of sap into the stelar region of shoots, depending on its concentration (Dong *et al.*, 2019; Kuriakose and Prasad, 2008). However, this transportation process, along with the detoxification and storage of Cd within plant cells, triggers anatomical and physiological changes that ultimately hinder the uptake of water and essential nutrients by the roots (Perez Chaca *et al.*, 2014). Studies by Nazar *et al.* (2012) have demonstrated a decrease in the concentration of essential minerals such as magnesium (Mg), calcium (Ca), and potassium (K) in various plant species including *Cucumis sativa*, *Lycopersicon esculentum*, *Lactuca sativa*, and *Zea mays*, attributed to Cd exposure.

Treatment with Cd resulted in a reduction of carbon content in the roots but a significant increase in the stems and leaves of *A. tenella* compared to control plants.

Carbon (C), a crucial macronutrient, constitutes about 50% of a plant cell's dry weight and is integral to various plant biomolecules such as carbohydrates, proteins, and nucleic acids. The observed increase in soluble sugar content in Cd-treated *A. tenella* leaves suggests a defence mechanism against Cd stress rather than a promotion of plant growth, as sugar translocation from leaves to roots is hindered by Cd, resulting in lower C content in roots (Li *et al.*, 2020). Furthermore, Cd toxicity induces full or partial stomatal closure, inhibiting carbon fixation and reducing photosynthetic activity upon continuous exposure to Cd (Haider *et al.*, 2021). Cd stress induced variations in the distribution of oxygen (O) in all three spectra studied in roots, stems, and leaves. Elevated O levels can enhance plant growth and development by facilitating nutrient uptake and cellular respiration. Potassium levels were significantly higher in the roots of Cd-treated *A. tenella* but slightly decreased in leaves. K⁺ ions play various roles in plants, including enhancing root-system proliferation, upregulating antioxidant enzyme activity, maintaining osmoregulation, and activating enzymes involved in metabolic pathways such as nitrogen (N) and C metabolism, sugar transport, photosynthesis, and protein synthesis (Xu *et al.*, 2020; Jia *et al.*, 2008). The increase in K assimilation in roots can be an act of tolerance imparted against Cd stress in the plant. K⁺ ions also regulate the opening and closing of stomata (Hasanuzzaman *et al.*, 2018); hence a decrease in K content in the leaves of Cd treated plants can be the reason for closed stomata and reduced photosynthetic activity observed in our study. Changes in the translocation of K and other nutrients to the shoot have been linked to alterations in the vascular system, including reductions in the numbers and diameters of xylem elements (Ouzounidou *et al.*, 1994). Phosphorus (P) content increased in the roots of Cd-treated plants compared to controls, possibly due to enhanced ATP synthesis from increased mitochondrial activity and antioxidant enzymes (Sarath *et al.*, 2022a). Increasing P concentration has been reported to reduce Cd mobility in plants, leading to its accumulation in roots rather than aerial parts (Ma *et al.*, 2022; Jia *et al.*, 2024), which is consistent with the bioaccumulation results obtained using ICP-AES in the present study. Interestingly, Ca was detected in control roots but not in Cd-treated plants, and vice versa for stem and leaf

samples. Calcium, known for its crucial role in signalling pathways, is readily absorbed by roots, translocates vertically, and induces stomatal closure upon reaching the leaves (Schroeder *et al.*, 2001). Studies indicate that Ca can alleviate Cd toxicity by neutralizing negatively charged plasma membrane surfaces, and potentially reducing the influx of Cd (Sarwar *et al.*, 2010).

Among several micronutrients, copper (Cu) and sodium (Na) were present only in the roots of Cd-treated plants, while Mg, aluminium (Al), silicon (Si), and sulphur (S) were present only in control roots. Zinc (Zn) and Cu were present only in control stems, while S was present in Cd-treated stems. Chlorine (Cl), Al, Zn, and Na were present only in control leaves, while iron (Fe) was observed only in Cd treated leaves. Cu content increased in Cd-treated leaves compared to controls. The increased S content in *A. tenella* shoots may be attributed to increased levels of phytochelatins or glutathione, which play a role in Cd stress tolerance (Gielen *et al.* 2017). The lower Zn content in the shoots of Cd treated plants compared to controls may be attributed to P-Zn interactions, where Zn deficiencies in leaves enhance P uptake rates by roots and its translocation to the shoots (Gomes *et al.*, 2013). Iron has been shown to alleviate Cd-induced oxidative stress in plants, as it serves as an essential cofactor for antioxidant enzymes like catalase and ascorbate peroxidase, thus maintaining high Fe levels in the roots during peak Cd concentrations can enhance the activity of these enzymes, serving as a crucial defence mechanism against ROS generated by Cd stress (Wang *et al.*, 2013; Sharma *et al.*, 2004). Thus, SEM-EDAX data indicate that the effect of Cd on the distribution and content of both macro and microelements in *A. tenella* varies depending on the plant parts.

The mechanism of Cr uptake in plants is not distinctly explained till date. In contrast to Cr (VI) which shares structural similarities with phosphate and sulphate and is therefore actively taken up by phosphate and sulphate transporters, plants take up Cr (III) through a passive process by diffusion at the cation exchange site of the cell wall (de Oliveira *et al.*, 2016; Singh *et al.*, 2013). The intake of essential minerals like Ca, Mg, P, and Fe has been shown to be reduced by excessive Cr, which masks the sorption sites and forms insoluble complexes (Basile *et al.*, 2012).

Treatment of Cr caused a reduction of C in the roots but marked an increase in leaves of *A. tenella* with reference to the control plants. The C content did not change significantly in the Cr treated stem from that of control. There has been a decrease in carbon absorption due to the insufficient ability of leaves to absorb CO₂ (Barthel *et al.*, 2011). This is related to the partially closed stomata due to Cr stress, which prevents the CO₂ needed for C fixation from entering the plant. The carbon that enters the leaf get reduced to sugars and this is correlated with the increased amount of soluble sugars in leaves of Cr treated *A. tenella*. But the translocation of these photoassimilates is reduced due to the Cr ions and hence there is less C content in the stem and roots compared to leaves (Mishra and Dubey, 2013). The distribution of O was also affected by the Cr treatment in *A. tenella*. The O content reduced in the treated plants compared to control in all plant parts. The increased activity of antioxidant enzymes is attributed to the higher level of K in the Cr-treated roots, and K also serves as a coenzyme or activator of several crucial enzymes (Mengel, 2007). The presence of K was not detected in the stem tissues of both control and treated plants, but the K content was reduced in the leaves of treated plants than the control plants. Similarly, P distribution was also increased in the roots of treated plants compared to control. Phosphorus content was detected in trace amounts in the leaf tissues of both the plant samples without any significant changes. The higher ATP synthesis due to increased mitochondrial activity and the activation of the antioxidant enzymes could account for the higher P level found in the roots of Cr-treated plantlets (Sarath *et al.*, 2022b). The Ca content was slightly increased in the Cr treated roots compared to control. As Ca²⁺ is an essential component of many signalling pathways, it is easily absorbed by roots and transported to shoots through xylem, controlling several physiochemical activities (Mansoor *et al.*, 2014; Tuteja *et al.*, 2007). Ca²⁺ has a significant influence on stomatal motility and regulates guard cell activity as a secondary messenger (Schroeder *et al.*, 2001). When plants are under metal stress, the lowering of Ca has a significant impact on how well the stomata function (Rucińska-Sobkowiak, 2016).

Of the several micronutrients, Fe, Al, Si and Cu, Zn were present in the roots and stem respectively of control plant roots in low quantities but in the treated roots,

these ions were not detected. S, Si and Fe were present only in the leaves of Cr-treated plants, but Na was present only in control sample. The changes in the distribution of Cu, Cl, Al, and Zn were not significant in the Cr treated plants compared to control. Thus, the SEM-EDAX microanalysis of roots, stem and leaves of control and Cr treated plantlets showed that Cr significantly affects the distribution and content of macroelements in *A. tenella*, but do not affect the content of micronutrients. Intracellular concentrations of the important nutrients manganese (Mn), P, Ca, Mg, K, and Fe in plants are altered by Cr (VI) (Gardea-Torresdey *et al.*, 2004). The findings of the present study can be corroborated with the reports from other plants such as *O. sativa* (Ahmad *et al.*, 2011), *Z. mays* (Mallick *et al.*, 2010), *Salsola kali* L. (Oliveira, 2012), and *Raphanus sativus* (Tiwari *et al.*, 2013).

The highest accumulation of Pb is observed in roots due to the presence of exodermis and endodermis, which serve as major apoplastic barriers and retention sites, minimizing the translocation of toxic metals to the shoots (Alves *et al.*, 2014). Roots absorb Pb via cation transporters (Kim *et al.*, 2002), and translocates it to the shoots through the apoplast/symplast. However, the transport, storage, and detoxification of metals in plant cells induce anatomical and physiological changes that impede the ability of roots to absorb water and essential nutrients (Perez Chaca *et al.*, 2014). Studies have shown a reduction in macro and micro-nutrient concentrations in various plant species under Pb stress, including *Triticum aestivum*, *Spinacia oleracea*, and *O. cubense* (Lamhamdi *et al.*, 2011; Alves *et al.*, 2014). Pb competes with essential elements and blocks their absorption sites on the roots, inhibiting uptake and translocation, thereby disrupting ionic homeostasis.

Pb treatment resulted in a reduction of C content in the roots, while an increment was observed in the leaves, and minimal changes were noted in the stem. Xiong *et al.* (2019) reported that under HM stress, plants increase the CO₂ concentration in leaves by enhancing their stomatal conductance to meet their respiratory needs. This leads to the accumulation of soluble sugars in leaves, as observed in Pb-treated *A. tenella* and hinders the translocation of sugars from leaves to roots, leading to a decreased C content in the roots (Li *et al.*, 2020). The reduction of O content in plant

tissues may be influenced by various factors like increased production of ROS, precipitation of Pb as carbonates, phosphates, and sulphates, and the binding of Pb to biological molecules. Potassium levels were higher in Pb-treated *A. tenella* compared to control plants, like the positive correlation between K concentration and Pb treatment found by Kibria *et al.* (2009) in *Amaranthus gangeticus* and *A. oleracea*. However, this observation contradicts findings in other plants reported by Lamhamdi *et al.* (2011), Akinci *et al.* (2010), and Kibria (2008). Phosphorus also showed a similar pattern to K in Pb-treated *A. tenella*, consistent with the results of Alves *et al.* (2014) in *O. cubense*. Both K and P play key roles in abiotic stress tolerance (Hasanuzzaman *et al.*, 2018; Khan *et al.*, 2023). Therefore, the increased assimilation of K and P in *A. tenella* may represent a tolerance strategy against Pb stress.

The increase in P concentrations in plant tissues may also result from Pb interfering with P metabolism or due to Zn deficiency (Gopal and Rizvi, 2008). Increased P content in shoots can also lead to precipitation of Pb as lead phosphate that occurs mainly in the cell wall and plant vacuoles, thereby serving as a strategy for Pb detoxification (Zheng *et al.*, 2012). Additionally, K^+ and P^{3-} ions are involved in stomatal regulation (Hasanuzzaman *et al.*, 2018; Khan *et al.*, 2023), which may explain the open stomata observed in the leaves of Pb-treated plants in our study. Calcium content showed fluctuations in different zones of *A. tenella* plant parts. Studies by He *et al.* (2020), Alves *et al.* (2014) and Lamhamdi *et al.* (2012) have reported a decrease in Ca^{2+} ions under Pb stress due to their ionic similarity that allows Pb to replace Ca during physiological processes. Pb may inhibit Ca absorption by either blocking Ca channels or through competitive transport, as observed in *O. sativa* (Huang and Cunningham, 1996; Kim *et al.*, 2002). Higher Ca concentrations in tissues under Pb stress may mitigate Pb phytotoxicity and increase plant tolerance by immobilizing Pb in the cell wall as reported in *Silene* and *O. sativa* (Antosiewicz, 1995; Malkowski *et al.*, 2002).

According to Kim *et al.* (2002), HMs can enter root cells through Mg^{2+} transporters and thus compete for the same carriers. Mg plays a critical role in chlorophyll

synthesis, photosynthetic assimilation, and sucrose transportation (Ishfaq *et al.*, 2022). The significant reduction in Mg content in Pb-treated *A. tenella* may explain its reduced pigment production and the impaired translocation of soluble sugars to the roots. Pb treatment also significantly decreased Zn content in *A. tenella*. This decrease in Zn content can be likely due to P-Zn interactions, where Zn deficiency disrupts membrane integrity and enhances the absorption and redistribution of phosphorus (P) in plant tissues (Kerbaudy, 2004). The increased S content in *A. tenella* shoots may be due to elevated levels of phytochelatins (PCs) or glutathione, which bind to metal ions like Pb facilitating their accumulation and detoxification (Kerbaudy, 2004). PCs help precipitate Pb as carbonates, phosphates, and sulfates (Salt *et al.*, 1998). S may also favor the sequestration of Pb in the form of PbSO_4 (Zheng *et al.*, 2012). *A. tenella* also showed an increase in the concentration of some nutrient ions such as Cl, Na, and Cu possibly due to the synergistic effects of Pb with these ions through various mechanisms (Kibria *et al.*, 2010). Changes in the micronutrient levels of *A. tenella* under Pb stress are consistent with findings in *O. cubense*, *A. gangeticus*, *A. oleracea*, cowpea, signal grass, and Rhodes grass (Alves *et al.*, 2014; Kibria *et al.*, 2009; Kopittke *et al.*, 2007a, b).

3.5.4. Biochemical and physiological studies

3.5.4.1. Relative Water Content

The negative effects of metal toxicity are more evident in aerial parts of the plant than in roots, making leaf relative water content (RWC) a potential indicator of the plant's ability to tolerate stress (Waheed *et al.*, 2022). The leaf RWC of Cd and Cr-treated *A. tenella* was found to be decreased compared to control plants. The decline in water uptake due to interaction of Cd^{2+} with aquaporin proteins may contribute to the reduction in RWC of treated plants (Shackira and Puthur, 2019). There were no significant differences in the leaf RWC of Cr- and Pb-treated *A. tenella* compared to control, indicating its tolerance to the metal. Water availability in plant cells is associated with the protoplasmic permeability of cells (Masuch *et al.*, 1998), and a loss of water content and nutrients from the cells would generally result in leaf senescence, which was not observed in *A. tenella* under Pb stress.

3.5.4.2. Photosynthetic efficiency

Photosynthesis is an important physiological process in plants which is severely affected during heavy metal stress. Studies have shown that Cd toxicity impairs the photosynthetic system inhibiting the fixation of carbon and the photosynthesis (Srivastava *et al.*, 2021; Ahanger *et al.*, 2020). Consistent with these findings, notable decrease in chlorophyll and carotenoid content was observed in *A. tenella*. This reduction correlates with a decline in plant dry weight, as photosynthesis contributes to biomass production (Li *et al.*, 2020). Reduction in chlorophyll content due to Cd stress has been observed in various plant species such as *Broussonetia papyrifera* and *Erigeron annuus* (Zhang *et al.*, 2020; Zhang *et al.*, 2021). The decline in chlorophyll content may be attributed to Cd replacing Mg in chlorophyll molecules or to increased chlorophyllase activity (Shackira and Puthur, 2019). Cd-induced degradation of photosynthetic pigments like Chlorophyll a and b reduces the net photosynthetic rate, correlating with dry biomass (Zhao *et al.*, 2021), which is consistent with the findings in *A. tenella*. Carotenoid levels in *A. tenella* showed a similar trend to chlorophyll under Cd stress. The decrease in carotenoid content induced by Cd may result from the inhibition of enzymes in pigment biosynthesis or from overproduction of reactive oxygen species (ROS) affecting carotenoid metabolic pathways (Qian *et al.*, 2009; Mishra *et al.*, 2006).

The total chlorophyll content of Cr treated *A. tenella* plantlets was increased initially, followed by a gradual decrease during the stress period. Chinmayee *et al.* (2014) had also reported similar findings in *J. curcas*. The degradation of photosynthetic pigments that cause deficiency in light-harvesting capacity is caused by exposure to high levels of heavy metals (Srivastava *et al.*, 2021). Gill *et al.* (2015) hypothesised that Cr at higher doses inhibits the activity of d-aminolaevulinic acid dehydratase, an essential enzyme in pigment synthesis that results in inhibiting chlorophyll biosynthesis. Studies on Cr stress in *V. radiata* (Singh *et al.*, 2021), *Sorghum bicolor* (Kumar *et al.*, 2019), *C. arietinum* (Singh *et al.*, 2020), *Z. mays* (Habiba *et al.*, 2019), and *Brassica oleracea* L. (Ahmad *et al.*, 2020) have reported similar results. Carotenoids also followed a similar trend to that of chlorophyll in *A. tenella*.

The decrease in carotenoids under Cr stress was also reported in *Phaseolus vulgaris* L. by Aldoobie and Beltagi (2013).

Under Pb stress, a non-significant reduction in chlorophyll and carotenoid content was observed in the tissues of *A. tenella*, suggesting that this plant may have better defence mechanisms against Pb toxicity, such as effective ROS scavenging and stronger pigment protection. This aligns with studies showing that some plants can maintain chlorophyll levels under HM stress (Yang *et al.*, 2011; Azmat *et al.*, 2009). The increased carotenoid content in Pb-treated *A. tenella* likely plays a crucial role in protecting chlorophyll from oxidative damage and degradation (Saadaoui *et al.*, 2022). According to Malik *et al.* (2021), plants may upregulate the biosynthetic pathways for chlorophyll and carotenoids in response to Pb exposure, keeping pigment levels stable despite the stress. Changes in the expression of stress proteins or genes related to pigment synthesis might also help maintain these levels (Malik *et al.*, 2021). The conditions of medium or physiological traits of the plant can limit Pb availability and absorption. If Pb uptake by the plants was lower than expected or if Pb was compartmentalized away from chloroplasts, this would reduce its direct impact on chlorophyll biosynthesis and carotenoid stability (Saadaoui *et al.*, 2022). *A. tenella* may also have such effective mechanisms to detoxify or sequester Pb ions, protecting chloroplasts and pigment biosynthesis pathways. This is supported by ICP-AES results showing more Pb concentration in the roots of *A. tenella* and less translocation to the leaves. According to Sharma and Dubey (2005), the photosynthetic rate is typically reduced by stomatal closure. However, SEM images showed high stomatal conductance in *A. tenella* leaves, which could explain the better photosynthetic pigment content in *A. tenella* even under Pb stress.

3.5.4.3. Total proteins

The total protein content of the root, stem and leaf samples of *A. tenella* after Cd and Cr treatment showed an increase initially, followed by a gradual decrease. A significant and rapid response of metal stressed plants is the induction of protein synthesis (Hall, 2002). The initial rise may be attributed to the synthesis of stress proteins such as heat shock proteins (HSPs), phytochelatins, metallothioneins, or

antioxidants like glutathione (GSH), which are known to protect plants from metal toxicity (Verma and Dubey, 2003; Mishra *et al.*, 2006). Metal stress induces an increase in proteins associated with transcriptional and translational control, antioxidant pathways, biosynthetic metabolism, and molecular chaperones, impacting proteins linked to food metabolism (Jain *et al.*, 2018). HSPs are significantly increased under conditions of metal toxicity to repair and stabilise the damaged proteins (Haap *et al.*, 2016; Pochodylo and Aristilde, 2017). Metal accumulation in plants triggers the release of phytohormones such as ethylene and jasmonic acid, as observed in *Phaseolus coccineus* (Pell *et al.*, 1997; Maksymiec *et al.*, 2005). Cd toxicity also stimulates the production of signalling proteins like MAPK, which in turn activate genes responsible for metal transport and degradation (Jonak *et al.*, 2004). An increase in total protein content under Cd stress has been reported in various plant species (Popova *et al.*, 2012; Lee *et al.*, 2010; Semane *et al.*, 2010). However, prolonged exposure to HMs can lead to protein fragmentation and degradation, affecting the structural and functional properties of proteins, thus exerting a detrimental effect on protein composition during extended growth periods (John *et al.*, 2009). Decreased protein synthesis in lettuce is attributed to increased protein degradation and reduced Rubisco activity (Monteiro *et al.*, 2009).

The increased total protein content under HM stress is typically shown by most plants due to the production of stress proteins and antioxidant enzymes (Lamhamdi *et al.*, 2011). However, Pb stress led to a notable reduction in total protein content across all plant organs of *A. tenella* throughout the treatment period. This corroborates with the findings on rice, Brassica, wheat, oat, barley, and *Nyctanthes arbor-tristis* (Awan *et al.*, 2015; Kumar *et al.*, 2019; Shehzad *et al.*, 2023; Souahi *et al.*, 2021). This decline can be attributed primarily to the activation of proteases under Pb stress. The increased protease activity accelerates protein breakdown, reducing the total protein levels in the plant (Palma *et al.*, 2002; Shehzad *et al.*, 2023). Pb stress also enhances the hydrolysis of proteins into their constituent amino acids more rapidly (Melnichuk *et al.*, 1982), and these amino acids tend to accumulate in the plant (Chen *et al.*, 2003). Additionally, protein content is lowered by the protein fragmentation and disintegration due to oxidative

stress caused by ROS generated by Pb exposure (Davies *et al.*, 1987; Kumar *et al.*, 2019). ROS-induced lipid peroxidation disrupts cellular membranes and directly damages proteins, causing them to lose functionality and undergo degradation (Palma *et al.*, 2002; Souahi *et al.*, 2021). Pb stress can interfere with normal protein synthesis, affecting the formation of crucial non-structural and functional proteins (Zheng *et al.*, 2021). This disruption is intensified by HM stress impairing ribosomal function, thus hindering new protein synthesis (Malik *et al.*, 2021). While Pb exposure may induce the synthesis of some stress proteins, their protective effects may not be sufficient to counterbalance the degradation of existing proteins leading to an overall decline in protein levels.

3.5.4.4. Soluble sugars

Sugars in plants play a dual role: they serve as metabolic reserves and structural components of cells while also regulating growth and development through signalling pathways. According to Sinha *et al.* (2017), characterizing the changes in soluble sugars and starch accumulation in leaves is essential for determining the phytotoxicity of HMs. Plants under stress increase their levels of soluble sugars. The plant tolerance to both biotic and abiotic stresses are depended on the accumulation and transport of these sugars derived from starch hydrolysis, and their distribution between various plant organs (Souahi *et al.*, 2021).

In Cd and Cr treated *A. tenella*, the soluble sugar content decreased gradually over the stress period after an initial spike. The initial rise in soluble sugar content may be attributed to the improvement in photosynthesis *and* increased carbon assimilation, serving as a defence response of the plant against osmotic and oxidative stress induced by Cd. Soluble sugars also play a crucial role in ROS scavenging, maintaining osmotic balance in cells, and stabilizing membranous structures under stress conditions (Wang *et al.*, 2021). Present findings regarding soluble sugars align with observations made in mung bean, Kentucky bluegrass and maize (Anwar *et al.*, 2021; Wang *et al.*, 2020; Li *et al.*, 2020). While sugars are primarily utilized for plant growth, they also play a crucial role in maintaining osmotic balance under severe osmotic stress conditions (Li *et al.*, 2020). The

decrease in soluble sugar content in *A. tenella* during later stages of stress could be attributed to reduced photosynthesis resulting from damage to the photosynthetic system and a decrease in leaf area (Armendariz *et al.*, 2016; Zhao *et al.*, 2021). The change in the soluble sugars and starch concentration of plants when treated with Cr was reported by Rodriguez *et al.* (2012), and Prado *et al.* (2010) and their results can be corroborated with the findings of the present study. The increase in soluble sugar content in stem and leaves of Pb-treated *A. tenella* during initial weeks aligns with findings from Malik *et al.* (2021), Souahi *et al.* (2021), and Khan *et al.* (2021), which suggest that *de novo* synthesis and reduced carbohydrate hydrolysis enhance sugar levels, aiding cellular protection under Pb stress.

The leaves of *A. tenella* exhibited higher levels of soluble sugars compared to the stem and roots under all metal treatments. This disparity may be due to impaired sugar translocation by Cd, leading to reduced transport of sugars from synthesizing sites (leaves) to sink tissues (roots) (Jha and Dubey, 2004; Mishra and Dubey, 2013). Additionally, the significant effect on soluble sugar content in leaves than in roots indicates the greater sensitivity of leaves to stress. Khan *et al.* (2021) noted that reduced chlorophyll levels due to Pb toxicity disrupt photosynthesis and cellular energy supply affecting soluble sugar synthesis. Sugar variations can alter the expression of genes involved in abiotic stress responses, such as SOD, HSPs, and glutathione-S-transferases (Price *et al.*, 2004).

Heavy metal-treated *A. tenella* exhibited significantly higher contents of proline, phenols, flavonoids, and malondialdehyde (MDA), consistent with earlier reports (Ahmad *et al.*, 2005; Hadi *et al.*, 2016). Significantly higher proline, phenol and malondialdehyde content was observed in *A. tenella* when subjected to HMs.

3.5.4.5. Proline

Accumulation of proline is one of the strategies plants adopt to counteract the toxic effect of HM stress (Clemens, 2006). It acts as an osmoprotectant, stabilizes membranes and proteins, scavenges reactive oxygen species (ROS), and chelates metals, thereby maintaining cellular homeostasis (Rai *et al.*, 2004; Hussain *et al.*,

2021; Hosseinifard *et al.*, 2022). According to reports, proline is the only amino acid that builds up in plant leaves under stress conditions. Proline accumulation in tissues leads to osmotic adjustment and serves as a dependent marker for genotypes associated with stress tolerance (Ganesh *et al.*, 2009). Proline synthesis is upregulated in leaves under stress conditions, resulting in higher concentrations compared to roots. Both biosynthesis and degradation by the mitochondrial enzyme proline dehydrogenase (PDH) contribute to proline accumulation in plant cells (Kavi-Kishor *et al.*, 2005). Interestingly, Lemzeri (2007) reported that tolerant plants typically show stable or low proline accumulation, which might explain the relatively low proline levels in *A. tenella* under Pb stress, suggesting tolerance to Pb as opposed to sensitivity towards Cr stress.

Elevated proline levels in response to heavy metal stress have been observed in various plant species, including *Medicago trunculata* and *Cucurbita pepo* under Cd stress (Garcia de la Torre *et al.*, 2022; Labidi *et al.*, 2021), Sorghum, sunflower, chickpea and maize (Singh *et al.*, 2020; Qadir *et al.*, 2020; Kumar *et al.*, 2019; Adhikari *et al.*, 2020) under Cr stress and black gram, wheat, and spinach (Yang *et al.*, 2011; Singh *et al.*, 2012; Lamhamdi *et al.*, 2011) under Pb stress.

3.5.4.6. Total phenolics

Elevated levels of phenolic compounds represent another defence mechanism of plants against heavy metal stress. Phenolics protect plant cells against HM stress by metal chelation, phenol-coupled ascorbate peroxidase activity and scavenging of ROS (Lavid *et al.*, 2001). Phenolic compounds protect plants from oxidative stress induced by metals through ROS scavenging and detoxifying hydrogen peroxide (H_2O_2) and induce anatomical modifications such as cell wall thickening and formation of physical barriers (Sakihama *et al.*, 2002; Michalak, 2006). The concentration of phenols in plant tissues is a valuable indicator of plant tolerance to various stress factors (Jańczak-Pieniążek *et al.*, 2023). The higher content of phenols in the *A. tenella* under HM stress aligns with previous studies on winter wheat, maize and tomato (Jańczak-Pieniążek *et al.*, 2022; Kisa *et al.*, 2016; Kisa *et al.*, 2019).

3.5.4.7. Total flavonoids

Plants show enhancement in their flavonoid content under heavy metal stress as a defence mechanism. The higher content of flavonoids observed in *A. tenella* under metal stress is consistent with previous studies (Dobrikova *et al.*, 2021; Jańczak-Pieniążek *et al.*, 2022). Flavonoids protect plants by scavenging ROS and chelating metal ions. Heavy metal exposure induces oxidative stress in plants by generating free radicals. Flavonoids are powerful non-enzymatic antioxidants that scavenge these ROS and hence prevents damage to biomolecules and stabilize cell membranes (Shomali *et al.*, 2022). The chemical structure of flavonoids, especially the presence of hydroxyl (-OH) groups, allows them to bind to and chelate metal ions. This forms stable complexes that reduce the toxicity and bioavailability of heavy metals like Cd^{2+} , Cr^{6+} , Cr^{3+} , and Pb^{2+} , and thereby protects plant cells (Mira *et al.*, 2002). Increased root growth, reduced lipid peroxidation and cell death are also reported in flavonoid-incubated plants under Pb toxicity (Izbiańska *et al.*, 2014). Previous studies on *V. radiata*, *Amaranthus hypochondriacus*, rice, and maize have shown increased flavonoid content under Cd, Cr, and Pb exposure (Sharma *et al.*, 2021; Li *et al.*, 2022; Kang *et al.*, 2024; Han *et al.*, 2024), suggesting their importance in plant defence against these heavy metals.

3.5.4.8. Malondialdehyde

Malondialdehyde (MDA) content represents the magnitude of lipid peroxidation as an important impact of HM toxicity in plants. The increased MDA levels in the root and shoot tissues at different concentrations of HMs are considered a sign of excess production of ROS oxidative damage or the due to external metal toxicity. Oxidative stress induced by HMs modifies proteins and carbohydrates, fluidity and permeability of membranes, and inactivates/damages nucleic acids (Malkowski *et al.*, 2019). Therefore, MDA concentration serves as a reliable indicator of physiological stress in plants. MDA content was increased in all parts of *A. tenella* under HM stress, consistent with previous findings in Cd-treated Citrus and strawberry (Giannakoula *et al.*, 2021; Muradoglu *et al.*, 2015), Cr-treated *C. arietinum* and maize (Singh *et al.*, 2020; Habiba *et al.*, 2019), Pb-treated *V. radiata*, *Pisum*

sativum, and *Coronopus didymus*, showing a consistent response to HM-induced oxidative stress across various plant species (Deshna and Bafna, 2013; Sidhu *et al.*, 2016; Dias *et al.*, 2019).

3.5.5. Antioxidant Enzyme Assays

A tissue-specific and temporal regulation in antioxidant enzyme responses reflecting the adaptive capacity of plant to cope with the oxidative stress was observed in *A. tenella*. Among the metals studied, Cr elicited the highest enzymatic induction followed by Cd and Pb generally had milder or delayed responses.

SOD catalyses the conversion of superoxide radicals ($O_2^{\bullet-}$) into H_2O_2 and oxygen, acting as the first line of defence against ROS (Sidhu *et al.*, 2016; Kumar *et al.*, 2019). SOD activity showed a mild upregulation consistently across tissues under all metal treatments in *A. tenella*, which suggests that HM exposure induced a moderate level of superoxide radicals. The enhanced scavenging of superoxide at the primary site of metal uptake was indicated by the pronounced induction of SOD in roots under metal stress. The moderate increase in SOD activity could be because the primary oxidative stress response is being managed by other antioxidant mechanisms or enzymes, like CAT and APX. Similar SOD induction has been reported in *Hibiscus syriacus*, *Canna orchoides* under Cd stress (Li *et al.*, 2023; Zhang *et al.*, 2021) and in maize under Cr stress (Anjum *et al.*, 2017). Pb elicited only slight SOD activity in *A. tenella* which allies with previous findings that Pb toxicity often disrupts enzymatic antioxidants through binding to sulfhydryl (-SH) groups rather than inducing strong oxidative bursts (Rahman *et al.*, 2024).

CAT is a major enzyme that reduces oxidative stress by scavenging hydrogen peroxide (H_2O_2) in peroxisomes and converting it into water and oxygen. CAT activity significantly increased in roots and leaves, particularly under Cr exposure. This indicates the vital role of CAT in detoxifying H_2O_2 generated by SOD activity. The higher increase in leaves might be due to higher metabolic activity and greater exposure to oxidative stress in photosynthetic tissues. The non-significant increase

in the stem suggests that the stem might have a lower demand for H_2O_2 detoxification or different mechanisms to cope with oxidative stress. A similar increase in CAT has been observed in *Brassica juncea* and *V. radiata* under Cr stress (Diwan *et al.*, 2010) and in rice under Cd stress (Yomso and Siddique, 2024). However, Pb induced only a mild increase in CAT activity compared to other metals, like previous observations in *Hemerocallis citrina* (Zhang *et al.*, 2024).

APX exhibited delayed but sustained responses in *A. tenella* and showed significant increases in roots and stems under Cd and Cr stress. This pattern suggests a phase-specific regulation of ascorbate-dependent H_2O_2 scavenging, complementing CAT activity during prolonged stress. The significant increase in APX activity in roots can be attributed to the roots being the primary site of Pb uptake and initial oxidative stress. Previous studies in castor, *C. orchoides*, *Monochoria korsakowii* under Cd (Srivastav *et al.*, 2024; Zhang *et al.*, 2021; Zhau *et al.*, 2022) and Sorghum, maize under Cr (Kumar *et al.*, 2022; Iftikhar *et al.*, 2025) also demonstrated strong APX activation. This highlights the importance of APX in maintaining the ascorbate-glutathione cycle under metal stress. Pb had least effect on APX activity in *A. tenella* which is in concordance with the earlier observations in *Typha angustifolia* (Bah *et al.*, 2011).

GPX reduces H_2O_2 to water using glutathione as a substrate and is crucial in mitigating oxidative stress in the cytoplasm and vacuoles. GPX displayed differential regulation depending on both plant tissue and metal. Cd and Cr significantly enhanced GPX activity in roots and leaves, supporting its role in maintaining glutathione-dependent redox balance. Similar increases were reported in castor, *Malva parviflora* under Cd (Srivastav *et al.*, 2024; Tousi *et al.*, 2020) and *Vigna mungo*, *Cucumis utillissimus* under Cr stress (Raj and Das, 2021; Sinam *et al.*, 2011). Pb inhibited GPX activity in roots and stems of *A. tenella*, while stimulating it in leaves. The decrease in GPX activity in roots and stems might be due the enzyme deactivation resulted from the binding of Pb to thiol groups (-SH) in proteins, leading to enzyme inactivation (Gupta *et al.*, 2009; Sharma and Dubey, 2005). The increase of GPX activity in leaves could be a compensatory mechanism to cope with

the elevated H_2O_2 levels generated due to increased SOD activity. Being photosynthetic tissues, leaves might require more robust antioxidant defences. This differential response is like observations in *Phaseolus vulgaris* and *Z. mays* (Geebelen *et al.*, 2002; Kaur *et al.*, 2015).

The tissue-specific responses of enzymes in *A. tenella* indicate a compartmentalized defence strategy that enables the plant to efficiently manage oxidative stress in both root and photosynthetic tissues. This strategic allocation of enzymatic defences highlights the higher tolerance of *A. tenella* to Cd and Cr stress compared to Pb and supports its potential as a candidate species for phytoremediation.

3.5.6. Fourier transform infrared (FTIR) analysis

FTIR profiling of *A. tenella* biomass serves as a tool for identifying potential plant metabolites involved in heavy metal binding. FTIR is a reliable method for identifying metal-induced changes to the structural composition of the biomolecules in root and leaf sample samples. The interactions between the transition metals Cd, Cr, Cu, and Ni and plant biomass are mostly mediated by amino sugars (Panda *et al.*, 2007). These sites primarily accommodate the cations H^+ , Na^+ , K^+ , Ca^{2+} , Mg^{2+} , and Fe^+ . Yet, there is a propensity for metal substitution when metals like Cd and Cr are present (Schneider *et al.*, 2001). Hence, carboxyl and amino groups are the means via which metals interact with *A. tenella* biomass. After the cationic exchange for metals via the functional groups, modifications to the structural moieties of lignin, cellulose, and proteins affect the growth pattern of plants, photosynthetic activity, and antioxidant system (Kumar *et al.*, 2017).

FTIR results shows that dry biomass has several functional groups such carboxyl, phosphate, amide, and hydroxide that are available for binding heavy metal ions (Usman *et al.*, 2019). Ion exchange via the carboxyl groups on the plant's surface is the primary mechanism for removing heavy metals. O-H and N-H stretch are characterised by the broad and robust IR spectral bands spanning $3600\text{--}3200\text{ cm}^{-1}$ (Panda *et al.*, 2007). All bands in the regions $2500\text{--}2000\text{ cm}^{-1}$ and $2000\text{--}1500\text{ cm}^{-1}$ corresponds to absorption caused by triple bonds and double bonds respectively.

All bands at the range of 1600 cm^{-1} corresponds to specific amide groups due to C=O stretch (Dumas and Miller, 2003). The regions from 1200 to 900 cm^{-1} signify C-C, C-O, and C-O-P stretch overlaps occurring mainly in cellular polysaccharides (Wolkers *et al.*, 2004).

Comparison of FTIR profiles between control and Cd-treated biomass revealed interactions between the metal and various functional groups such as O-H, N-H, C-H, $\text{C}\equiv\text{C}$, C=O, P=O, -C-OH, and C-S present in different metabolites. This result is in conformity with previous studies (Bora and Sarma 2021; Su *et al.*, 2017). The peaks and their corresponding shifts due to metal interaction in the plant parts of *A. tenella* that was found to be consistent in all tissues were studied in detail. After exposing to Cd for 21d, the absorption peak at 3412 cm^{-1} for root control (RoC) representing the hydroxyl group (-OH) corresponding to phenols and alcohols shifted to a higher frequency region (3419 cm^{-1}) in treated roots (RoT). In stem, absorption peak at 3419 cm^{-1} for control (SoC) was shifted to 3423 cm^{-1} for stem treated (SoT). The interaction of Cd^{2+} ions with the aliphatic primary amine group for metal oxygen binding produces these shifts. RoC showed a band at 1641 cm^{-1} shifting to 1639 cm^{-1} for RoT, from 1640 cm^{-1} for SoC to 1635 cm^{-1} for SoT, 1627 cm^{-1} for LoC to 1638 cm^{-1} region for LoT. These shifts were due to the cationic interaction of Cd with carbonyl groups (ketones/aldehydes). There were decrease in bands intensity from RoC at 1060 to 1052 cm^{-1} for RoT, SoC from 1054 to 1057 cm^{-1} for SoT, LoC from 1060 at 1051 cm^{-1} LoT. Strong metal binding with C-C, C-O, and C-O-P causes these shifts (Sheng *et al.*, 2004). The band shifts correspond to the symmetric bending vibration of C-H and -COOH mainly from hemicellulose, cellulose and pectin on the cell wall on interaction with metal ions (Sruthi and Puthur, 2019). A major shift in the absorption peak was recorded from 2145 cm^{-1} for RoC to 2125 cm^{-1} for RoT which characterizes the $\text{N}=\text{C}=\text{N}$ (carbodiimide) stretch, which is important for the preparation of many functional groups including carboxylic acids.

Although, three to five major bands were found to correspond to Cr interaction in the root, stem and leaf samples of *A. tenella*, only three of these peaks, and their

corresponding shifts were consistent on comparison to all tissues, and hence studied in detail. Examining FTIR results, root control (RoC) showed a band at 3412 cm^{-1} shifting to 3418 cm^{-1} for root treated (RoT), from 3419 cm^{-1} for stem control (SoC) to 3421 cm^{-1} for stem treated (SoT), 3423 cm^{-1} for leaf control (LoC) to no peak at $3200 - 3600\text{ cm}^{-1}$ regions for leaf treated (LoT). These band shifts are due to cationic interaction of Cr^{2+} with the hydroxyl group for metal oxygen binding. Similar pattern was noted by Usman *et al.* (2019) when *Tetraena qatarensis* was assessed for the accumulation of heavy metals including Cr, D'Souza (2008) when *Padina tetrastrum* Hauck was treated with and without Cd and Panda *et al.* (2007) following Cd and Ni adsorption by *Lathyrus sativus* L. RoC showed a band at 1641 cm^{-1} shifting to 1638 cm^{-1} for RoT, from 1640 cm^{-1} for SoC to 1638 cm^{-1} for SoT, 1627 cm^{-1} for LoC to 1641 cm^{-1} region for LoT. These shifts are due to the interaction of Cr^{2+} ions with specific amide groups due to C=O stretch. There were decrease in bands intensity from RoC at 1060 to 1054 cm^{-1} for RoT, SoC from 1054 to 1055 cm^{-1} for SoT, LoC from 1060 to 1071 cm^{-1} LoT. These shifts can be attributed to strong metal binding with C-C, C-O, and C-O-P (Sheng *et al.*, 2004). A prominent band shift was observed at RoC showing a band at 1360 cm^{-1} shifting to 1381 cm^{-1} for RoT, which corresponds to CH_3 deformation. This indicates the enhancement of hemicellulose in xylem cell walls that lead to increase in its thickness (Sarath *et al.*, 2022a).

In Pb-treated samples, the increased intensity in the $3200\text{-}3600\text{ cm}^{-1}$ region indicates enhanced interactions between Pb ions and hydroxyl groups, consistent with prior studies where O-H groups play a crucial role in metal ion binding and complexation (Wang *et al.*, 2016). Changes observed around $2600\text{-}2950\text{ cm}^{-1}$ suggest alterations in lipid metabolism and hydrocarbon chains, reflecting the plant's adaptive response to Pb stress. Plants often secrete more organic acids to resist metal toxicity, enhancing the carboxylic acid bands and promoting the binding of heavy metals to polyester polysaccharides. This mechanism, as noted by Su *et al.* (2022) and Chandrasekhar and Ray (2019), may explain the lack of significant changes in the growth parameters of *A. tenella*. Shifts around 1600 cm^{-1} indicate that Pb may bind to protein amide groups, potentially altering protein structures and

functions. Roots also exude amino acids, peptides, and proteins in response to Pb toxicity. The presence of Pb can stimulate or inhibit the secretion of these compounds, affecting the plant's ability to complex and mitigate Pb toxicity (Wang *et al.*, 2016). Fig. 3.26A shows that, apart from irregular shifts at the 2132 cm^{-1} peak, there were no significant changes in the remaining peaks. This stability suggests that organic components remained relatively unaffected by Pb stress, indicating that the physiological and biochemical processes of *A. tenella* were minimally impacted. This implies strong Pb tolerance in *A. tenella*, likely due to the impediment of Pb translocation in the stem. Heavy metals can induce the synthesis of protective proteins, such as proline-rich, disease-related, and histidine-rich proteins, which help protect plant cells from heavy metal toxicity (Gu *et al.*, 2009; Bosch *et al.*, 2006). The increase in the amide bending vibration peak near 1640 cm^{-1} suggests increased production of amino acids, peptides, and proteins in *A. tenella* leaves under Pb stress. The disappearance of peaks at 1316.42 cm^{-1} and 1241.11 cm^{-1} in Pb-treated samples, compared to controls, indicates degradation or modification of cellulose and lignin structures in the plant leaves, suggesting a protective mechanism to reduce Pb toxicity by disrupting cell wall integrity. Changes in the $1200\text{-}900\text{ cm}^{-1}$ region suggest that Pb binding involves C-C, C-O, and C-O-P stretches, affecting the plant's cell wall composition and structure. In the $700\text{-}400\text{ cm}^{-1}$ region, the spectral changes further support the hypothesis that Pb interacts with hydroxyl groups in the cell wall. Compared to the control, Pb-treated *A. tenella* biomass shows significant fluctuations in peak intensity, with some peaks either increasing or disappearing. These spectral changes can be attributed to increased Pb content, which alters the absorbance characteristics of the biomass. The enhanced peak intensities observed in the roots suggest that they serve as the primary site for Pb accumulation, limiting its translocation to aerial parts, aligning with findings from similar studies where root barriers help mitigate heavy metal transport and protect aerial tissues (Wang *et al.*, 2016; Su *et al.*, 2022; Peng *et al.*, 2020).

Finally, FTIR results showed a consistent decrease in band intensity of RoT as compared to SoT under all metal treatments that was comparable with the ICP-AES

result, which showed that *A. tenella* predominantly adsorbs metals (Cd, Cr, and Pb) in the root with subsequent translocation to the stem and leaves. Wang *et al.* (2009) has reported the tenfold higher concentration of Cd in plant roots than the aboveground tissues that indicates the role of roots as a barrier in Cd translocation, and this efficacy of the roots is attributed to the functional groups available in the cell wall like hydroxyl, carboxyl groups that interact and form complexes with the heavy metals (Shah *et al.*, 2019; Nishizano *et al.*, 1987).

3.5.7. Heavy metal biodistribution in plant tissues

An effective phytoremediation plant must be able to produce biomass even under stress. The metal accumulation in biomass, BCF, BAC, and TF of the plants determines their potential for phytoremediation (Abou-Aly *et al.*, 2021; Parihar *et al.*, 2021; Budzynska *et al.*, 2019). Typically, HM concentration in plant parts follows the pattern: roots > stem > leaves (Ahmadpour *et al.*, 2015). Roots are the primary organs to encounter HMs in the growth medium. However, to mitigate damage to plant tissues and the photosynthetic apparatus caused by these metals, plants often restrict HM transport to shoots and accumulate it in root cell walls and vacuoles (Hanikenne *et al.*, 2011). This serves as a defence mechanism to protect above-ground tissues and enables plants to tolerate Cd toxicity.

In our study, the concentration of Cd was observed to be highest in the roots of Cd treated *A. tenella* (317.25 mgkg⁻¹ DW) followed by 87.462 mgkg⁻¹ DW in the stem and 72.825 mgkg⁻¹ DW in leaves. Due to its high mobility, Cd is readily absorbed by roots and transported to shoots through the xylem. The higher HM concentration in roots compared to aerial parts of the plant is attributed to low translocation indices and Cd accumulation in cell walls and vacuoles (Zhang *et al.*, 2022; Pereira *et al.*, 2018). The elevated Cd accumulation in root tissues suggests the potential of *A. tenella* for phytostabilization of Cd. Similar observations have been made in *Acacia mangium*, *Jatropha curcas*, and *Manihot esculenta* (Taeprayoon *et al.*, 2022).

In the present study, a higher accumulation of total Cr was also observed in the roots of *A. tenella* exposed to Cr treatment (179.625 mg/kg DW) and the

accumulation of Cr in the stem (87.075 mg/kg DW) and leaves (98.625 mg/kg DW) were less compared to roots. Similar observations were found in *Diectomis fastigiata* (Sw.) P.Beauv. (Mohanty and Patra, 2020), *Melia azedarach* (Yan *et al.*, 2020), *V. radiata* (Singh *et al.*, 2021), *B. oleracea* (Ahmad *et al.*, 2020), *C. arietinum* (Singh *et al.*, 2020), and *Arachis hypogaea* (Zong *et al.*, 2020). Sometimes, the root concentration of Cr exceeds the shoot concentration by 100 times (Shanker *et al.*, 2005). This could be due to the low mobility of Cr in the plant roots compared to other metals or the sequestration of Cr in the vacuoles of root cells which acts as a protective mechanism and provides natural tolerance to plants against Cr toxicity (Mangabeira *et al.*, 2011). Stress ethylene is induced by Cr in plant roots that cause strong cellular damage and is transported to shoots from roots. Since the root encounters stress ethylene prior to shoot, the roots get damaged first (Shinwari *et al.*, 2015; Shahid *et al.*, 2017). The transport of Cr also depends on its oxidation state. The conversion of Cr (VI) to Cr (III) in plant tissues prevents further transport of Cr because Cr (III) has a propensity to attach to cell walls (Kabata-Pendias and Szteke, 2015). The plants such as *Dyera costulata* (Gafoori *et al.*, 2013), *Azolla caroliniana* (Pandey, 2012), *Amaranthus dubius* (Mellem *et al.*, 2012) showed a significant translocation of Cr from roots to aerial parts and thus, suggested that these species have a high phytoremediation potential.

A. tenella preferentially concentrates Pb in the root (2682.5 mgkg⁻¹ DW) followed by 324.75 mgkg⁻¹ DW in the stem and 212.375 mgkg⁻¹ DW in leaves. Only about 20% of Pb in the roots is translocated to the shoots, which creates a barrier to HM translocation and restricts its transfer to the aerial part of the plants, as seen in species such as *T. qatarense*, *Z. mays*, *Cyamopsis tetragonoloba*, and *Sesamum indicum* (Usman *et al.*, 2020; Liu *et al.*, 2020; Amin *et al.*, 2018). Kumar *et al.* (2017) found that in most plant species, about 95% of the absorbed lead is retained in the roots, with only a small fraction reaching the aerial plant parts. In the present study, 78.81% of the total Pb was accumulated in the roots, while 21.19% was in the shoots. Plant roots passively absorb free Pb²⁺ ions from the medium along with the nutrients, moving them through the root apoplast and radially across the cortex. These ions are then transported upward through the xylem and released into the endoderm

(Sharma and Dubey, 2005). Additionally, Pb ions from polluted air can be absorbed through the cuticle and stomata in the leaves, potentially leading to chlorosis and reaching the endodermis where the ions bind to the cell wall and plasma membrane. However, the Casparian strip in the endodermis and exodermis restricts this radial transport of Pb in the root and shoot, reducing Pb translocation and protecting the aerial tissues and photosynthetic apparatus (Morel *et al.*, 1986, Seregin *et al.*, 2007).

Bioconcentration factor (BCF) and translocation factors (TF) are two important parameters used to evaluate the potential of a plant to remediate a particular metal (Sinha *et al.*, 2014). BCF and TF values can be used to assess the ability of plants to translocate heavy metals from soil to plant parts and from root to shoot respectively (Siyar *et al.*, 2022). Bioaccumulation coefficient (BAC) measures metal tolerance and accumulation in total plant biomass. These values quantify the bioavailability and mobility of metals, and the ability of plants to accumulate metals in the roots and shoots (Castro-Bedriñana *et al.*, 2021). The plant with both BCF and TF values greater than 1 is a potent candidate for phytoextraction, while plants with BCF values greater than 1 and low TF ($TF < 1$) and BAC levels indicate phytostabilization potential (González-Chávez and Carrillo-González, 2013). *A. tenella* exhibited a BCF_{root} value >1 but $TF < 1$ for Cd and Pb, supporting its ability for Cd and Pb phytostabilization. But in case of Cr, *A. tenella* showed a very high BCF_{root} value (>1) and $TF > 1$, and this substantiates the phytoextraction potential of *A. tenella* towards Cr. The higher BAC of Cd and Pb-exposed *A. tenella* indicates its tolerance and Cd accumulation capacity, especially in roots.

Mesembryanthemum crystallinum and *Lemna minor* are plants reported for their phytoextraction potential of Cr (Sliwa-Cebula *et al.*, 2020; Sallah-Ud-Din *et al.*, 2017). Plants that can absorb heavy metal ions more than 100 times that of ordinary plants, and whose leaf-to-root ratio (TF) of HM content is > 1 are called hyperaccumulators (Baker *et al.*, 2000; Saud *et al.*, 2022). 300 $\mu\text{g/g}$ is the plant absorption threshold for Cr (Van Der Ent *et al.*, 2013). Several species have been recently identified as hyperaccumulators of Cr, like *Cannabis sativa* and *Allium*

griffithianum (Sajad *et al.*, 2020), *Cirsium vulgare* (Dökmeci and Adilo, 2020), *Ipomoea aquatica* (Haokip and Gupta, 2020), *Cassia tora* (Patra *et al.*, 2021), and *Eichhornia crassipes* (Mondal and Nayek, 2020). However, existing hyperaccumulator plants have slow growth, low biomass or economic benefits, so they cannot be practically used for remediation. The plant under study, *A. tenella*, being an invasive fast-growing plant with $TF > 1$ thus meet the criteria for a phytoextractor plant and can be a hyperaccumulator, if the concentration of Cr translocation to shoot can be enhanced.

Heavy metal stress induced significant phenotypic and physiological alterations in *A. tenella* plantlets. Notable reductions in the elongation of root and shoot, leaf expansion and overall biomass accumulation in metal-treated plantlets indicates the impaired growth dynamics under metal toxicity. Anatomical modifications such as thickening of xylem walls, reduction in xylem diameter, and evident distortion in root and stem structure underscore the impact of metal stress on plant morphology. Elemental profiling revealed altered distribution patterns of macro and micro-elements within the plant tissues, reflecting disturbances in nutrient uptake and allocation. Biochemical assays indicated reduced chlorophyll content and simultaneous accumulation of stress-related metabolites like proline and malondialdehyde. Moreover, differential metal accumulation patterns were observed, with roots exhibiting higher metal uptake compared to stems and leaves, suggesting a potential role for phytostabilization mechanisms. These findings highlight the comprehensive effects of metal stress on *A. tenella*, impacting growth, anatomy, physiology, and biochemistry.

Due to its rapid growth, invasive nature, high BCF and BAC values, and limited economic utility, *A. tenella* is an ideal candidate for phytoremediation of areas contaminated with Cd, Cr, and Pb. While it exhibits phytostabilization of Cd and Pb, the plant efficiently phytoextracts Cr that highlights a unique and efficient mechanism for Cr detoxification. To elucidate the underlying molecular processes responsible for Cr tolerance and accumulation, the following chapter explores the transcriptomic and metabolomic responses of *A. tenella* under chromium stress.

4.1. Introduction

Chromium (Cr) contamination is an alarming environmental issue in industrial zones and riparian ecosystems exposed to untreated effluents. Among its various forms, hexavalent Cr [Cr(VI)] is highly toxic and carcinogenic. It has high mobility in soil and water systems. Cr alters the morphological, biochemical, and physiological processes in plants upon entering the plant tissues, even though it has no specific role in plant metabolism. Cr toxicity evokes a series of responses to plants like chlorosis, necrosis, wilting, and retardation of growth and development. Cr inhibits the nutrient absorption and distribution in plant tissues, biosynthesis of photosynthetic pigments, seed germination, root development, nitrogen assimilation, and disturbs water relations. The rapid production of ROS by Cr induces oxidative stress in plant tissues and alters both enzymatic and non-enzymatic antioxidant systems. The accumulation of Cr in plants leads to the entry of Cr into the animals and humans through the food chain and has serious effects like lung cancer, gastro-intestinal irritations, allergic reactions, ulcerations, and skin diseases (Kapoor *et al.*, 2022). The persistence of Cr in the environment and its potential entry into the food chain necessitate effective remediation strategies. Phytoremediation can be employed as a sustainable method for the remediation of Cr-contaminated area to prevent the accumulation of Cr in the trophic levels.

While the physiological, morphological, and structural responses of plants to Cr stress have been extensively studied, the molecular studies have been limited to model plants and a few related genera. Molecular mechanisms underlying the transcript levels in response to metal stresses remain largely unknown in plants. Heavy metal tolerance and detoxification involve the action of multiple molecular processes and networks, including the signalling pathways and changes in gene and metabolite expression that are not visible at the whole-plant level. A detailed understanding of these molecular responses is essential to identify key regulatory genes, stress-adaptive metabolites, and detoxification pathways that imparts metal tolerance in plants. Hence, studying the functional roles of genes and metabolites in

Cr-stressed plants is important for understanding the molecular basis of Cr tolerance and detoxification.

In recent years, advances in “Omics” technologies have enabled better understanding of the physiological and molecular mechanisms behind plant adaptation and tolerance to abiotic stresses including those induced by heavy metals. Transcriptomics and metabolomics have emerged as powerful tools for identifying genes and metabolic pathways involved in stress responses in plants. These techniques enable expression profiling and metabolite analysis during stress exposure and adaptation. Next Generation Sequencing (NGS) based RNA sequencing (RNA-seq) is a robust approach for transcriptomic studies which enables gene level studies in different tissues, conditions and developmental stages (Geng *et al.*, 2011). Transcriptomics allows an extensive analysis of changes in gene expression under metal stress and helps to identify the differentially expressed genes (DGE) involved in different processes such as metal transport, redox regulation, and secondary metabolism. Simultaneously, untargeted metabolomics reveal the dynamic shifts within plant cells to show how primary and secondary metabolic pathways adjust to support detoxification and stress adaptation. It also helps to identify and quantify different metabolites present in plants. As a downstream process of genomics and proteomics, metabolomics has the closest link to the phenotype of an organism (Zhang *et al.*, 2019). Thus, combining transcriptome sequencing (RNA-Seq) and metabolomic analyses provides an effective understanding of the molecular mechanism of gene and metabolite mediated heavy metal resistance in plants.

Various omics-based studies have provided the different response mechanism against Cr stress in different plants such as *Canna indica* (Zhu *et al.*, 2024), *Arabidopsis thaliana* (Liu *et al.*, 2020), and *Helianthus annuus* (Ibarra *et al.*, 2019), *Raphanus sativus* (Xie *et al.*, 2015), and *Oryza sativa* (Dubey *et al.*, 2010). Molecular level studies remain limited in *A. tenella* despite showing potential as a phytoremediator due to its strong tolerance and growth in contaminated areas. No transcriptomic or metabolomic data regarding the mechanisms of Cr uptake,

detoxification, and stress response are available to date. This knowledge gap limits the understanding of its detoxification potential and the broader applicability of this species in phytoremediation efforts. This study aims to fill this gap by using an integrated omics approach to explore the Cr tolerance in *A. tenella* by gene to metabolite networks. The objectives included the identification of key genes, metabolites and pathways involved in Cr detoxification and stress response in *A. tenella* leaves under Cr stress using RNA-seq and LC-MS. The findings are expected to provide novel insights into Cr tolerance mechanisms in *A. tenella* and support phytoremediation research in non-model species.

4.2. Review of Literature

Heavy metal pollution poses a significant threat to environmental and agricultural sustainability, with hexavalent chromium [Cr(VI)] being one of the most toxic and persistent contaminants. Many studies have examined the morphological and physiological responses in plants under Cr stress. However, understanding the molecular and metabolic mechanisms behind these responses is important for developing effective phytoremediation strategies. High-throughput technologies such as transcriptomics and metabolomics now make this possible. These methods help explain heavy metal detoxification in plants in terms of modulations in gene expression and biochemical pathways. The present study uses present knowledge of transcriptomic and metabolomic research on Cr and other heavy metal stresses in plants to explain the molecular mechanism of Cr detoxification in *A. tenella* which is a promising candidate for phytoremediation.

Plants use several strategies to cope with heavy metal stress. These include metal uptake, chelation, vacuolar sequestration, activation of antioxidant systems, and changes in signaling and metabolic pathways. Chromium detoxification involves extracellular and intracellular mechanisms. Extracellular processes include root surface adsorption and binding to the cell wall components. Intracellular mechanisms involve complexation with ligands such as phytochelatins and metallothioneins, reduction of toxic Cr(VI) to less toxic Cr(III), and compartmentalization of Cr inside vacuoles through membrane transporters.

4.2.1. Metabolomic responses in plants to heavy metal stress

Heavy metals (HMs) enter plants mainly through the roots and induce various physiological, biochemical and molecular responses to reduce their toxicity. The first line of defense is the cell wall which acts as a physical and chemical barrier restricting metal entry. Polysaccharides in the cell wall contain functional groups like carboxyl, hydroxyl and phosphate groups that can bind and immobilize heavy metal ions, which prevents them from entering the cell cytoplasm and protects cellular components. This cell wall remodeling is an important response to heavy metal stress and involves cellulose and hemicellulose families such as mannoglycan, xyloglycan, and xyloglucan. Soluble carbohydrates also function as signaling molecules and osmoprotectants (Cheng *et al.*, 2025).

Among phenolic compounds, ferulic acid and caffeic acid play important roles in reinforcing the plant cell wall and restricting heavy metal translocation. Ferulic acid is a natural phenolic compound synthesized through phenylpropanoid pathway. It is a potent antioxidant, scavenges free radicals and protects the cellular components from oxidative stress damage under abiotic stress (Khan *et al.*, 2024). The chelating properties of ferulic acid allows it to bind to metal ions like Cu and Fe to reduce their toxicity. It is also involved in cross-linking components of the plant cell wall which strengthens the structure and acts as defense against heavy metal entry (Khan *et al.*, 2024; Šamec *et al.*, 2021). Similarly, caffeic acid and its derivatives enhance tolerance to heavy metal stress in plants through their strong antioxidant and metal-chelating properties. They are precursors for lignin biosynthesis and thus play a role in reinforcing cell walls and restricting metal translocation. Studies have reported the alteration in the levels of caffeic acid and its esters under heavy metal exposure in plants. While they showed upregulation in wheat (Jańczak-Pieniążek *et al.*, 2022), the levels of caffeic acid along with ferulic acids were decreased in maize (Kisa *et al.*, 2016).

Once metals enter the cell, plants use several chelating and sequestering mechanisms to detoxify and compartmentalize metal ions. Phenolic compounds such as flavonoids are secondary metabolites that play an important role in this

process. Flavonoids in plants are classified into several subclasses including flavonols (such as quercetin, fisetin, myricetin, and kaempferol), flavanones (like naringenin and hesperetin), flavones (e.g., apigenin, luteolin), isoflavones, isoflavonoids, catechins and anthocyanidins (Singh *et al.*, 2021). These compounds play role in metal chelation and ROS scavenging to protect plants from oxidative damage. Several phenolic compounds including p-coumaric acid, ferulic acid, quercetin, epicatechin, vanillin and myricetin were localized in the root cell wall of *Zea mays* under heavy metal stress (Vuletić *et al.*, 2014). Quercetin is a subclass of flavonoids, which is a powerful antioxidant and hence plays an important role in various biochemical and physiological processes to provide plant tolerance against different stress conditions including heavy metal stress. Aslam *et al.* (2022) reported the role of quercetin in alleviating Cr stress in *Trigonella corniculata* by augmenting the activity of antioxidant enzymes and photosynthetic system and by reducing the concentration of stress markers including H₂O₂. Quercetin and its derivatives have multiple hydroxyl groups that allow it to form complexes with toxic metal ions such as Pb²⁺, Fe²⁺, Al³⁺, Zn²⁺ and Cu²⁺ (Eno *et al.*, 2023). This binding effectively reduces the bioavailability of metal ions, inhibits ROS generation and thus act as efficient metal chelators and ROS scavengers (Singh *et al.*, 2021). In *A. thaliana*, both quercetin and naringenin had reversed the harmful effects of Cd²⁺ and Zn²⁺ ions (Keilig and Ludwig-Müller, 2009).

Jan *et al.* (2022) reviewed that the glycosides of quercetin and kaempferol, including quercetin 3-O-rhamnoside 7-O-rhamnoside, quercetin 3-O-[6''-O-(rhamnosyl) glucoside] 7-O-rhamnoside, quercetin 3-O-glucoside 7-O-rhamnoside, kaempferol-3,7-dirhamnoside, kaempferol 3-O-[6''-O-(rhamnosyl) glucoside] 7-O rhamnoside and kaempferol 3-O glucoside 7-Orhamnoside have important roles in tolerating abiotic stress in plants. Quercetin and Kaempferol increased the activity of chloroplastic antioxidant enzymes and protected photosynthetic reactions from oxidative damage in wheat leaves under As stress (Arikan *et al.*, 2023). Quercetin levels were higher in maize roots exposed to aluminium, suggesting its ability for metal chelation as an in vivo mechanism to reduce Al toxicity (Kidd *et al.*, 2001). Manan *et al.* (2015) detected the enhanced accumulation of myricetin and

kaempferol *Nephrolepis biserrata* grown in heavy metal-contaminated soil. Rutin is another flavonoid of which the metal chelating activity is reported (Soczyńska-Kordala *et al.*, 2001). Rutin, luteolin and apigenin were found in arsenic hyperaccumulator fern *Pteris multifida* (Lu *et al.*, 1999). Rutin, quercetin, hyperin, kaempferol and total flavonoids were enhanced under As treatment in *P. multifida* and *P. semipinnata* (Wang *et al.*, 2010).

The flavonoid, Calendoflaside has been reported with potential to mitigate heavy metal stress such as Fe, Cu, Mn, Ni, Co, Zn, Cd, Mg, Ca, and As in *Calendula officinalis* (Varshney *et al.*, 2023). Isorhamnetin and its glycosylated forms also help plants to combat heavy metal stress in plants mainly due to its antioxidant properties. This is achieved by direct neutralization of free radicals, the upregulation of endogenous antioxidant enzymes like SOD, GPX and CAT or the activation of Nrf2 pathway (Khaled, 2020). Heavy metal exposure also influences the synthesis of specific flavonoids depending on the type of metal. For example, exposure to mercury increased the levels of caffeic acid, ferulic acid, chlorogenic acid, rosmarinic acid, *p*- coumaric acid, while copper toxicity enhanced the production of luteolin and apigenin in *Bellis perennis* (Karakas *et al.*, 2022). Apigenin production in *Matricaria chamomilla* enhanced with increased concentration of Cd, attributed to its antioxidant and sequestering property as a tolerance response to excess Cd (Zarinkamar *et al.*, 2021).

Auxin and indole compounds play important roles in modulating plant responses to heavy metal toxicity within the cytoplasm. The primary auxin, indole-3-acetic acid is synthesized from the amino acid tryptophan and is involved in cross talk with other plant hormones to generate response to heavy metal stress (Yang and Wang, 2023). Mild heavy metal stress can trigger the production of IAA, while severe HM stress can damage the auxin metabolic cycle leading to decreased IAA levels and impaired growth (Rahman *et al.*, 2023; Yang and Wang, 2023). Salicylic acid (SA) is a simple phenolic compound, which interacts with other plant hormones under heavy metal stress and promotes the stimulation of antioxidant enzymes (Sharma *et al.*, 2020). Various heavy metals including Cd, Pb, As, and Cr

induced the production of endogenous SA which improved the growth and development and mitigated stress responses in plants such as *Solanum tuberosum*, *Artemisia annua*, *Oryza sativa*, *Sorghum bicolor*, and *Brassica napus* (Sharma *et al.*, 2020).

Phosphatidylcholine (PC) is a primary component of the phospholipid bilayer of cell, which is involved in membrane integrity and homeostasis, stress signaling, antioxidant defense, and heavy metal accumulation (Sun *et al.*, 2022; Zhang *et al.*, 2025; Akpinar and Cansev, 2024). Exogenous PC increased the activity of antioxidant and non-antioxidant enzymes under lead stress in maize (Zhang *et al.*, 2025). PC and its precursor choline have been shown to reduce the uptake and accumulation of heavy metals in plant tissues. Exogenous choline reduced the Cd and Pb content in tomato and maize respectively and hence prevents the inhibition of plant growth due to metal toxicity (Zhang *et al.*, 2025; Akpinar and Cansev, 2024). This effect on metal uptake is partly due to the stabilization of root cell membranes by PC, limiting the passage of metal ions.

Heavy metals damage the structure of chloroplasts, inhibit chlorophyll biosynthesis, and accelerate chlorophyll degradation (Hourri *et al.*, 2020). Pyropheophorbide a (PyroPA) is a chlorophyll degradation product whose concentration can increase in plants under heavy metal stress, indicating the breakdown of the photosynthetic apparatus. Increased PyroPA reflects chlorophyll destruction and photooxidative stress due to metal toxicity, as reported under Pb and Cd stress on mulberry (Huihui *et al.*, 2020). Exogenous application of methyl jasmonate reduced Cr-induced damage in *C. officinalis* by enhancing the antioxidant defense system, maintaining the ascorbate-glutathione pool, activating glyoxalase enzymes and improving photosynthetic pigments (Barzin *et al.*, 2022). Apo-10'-violaxanthal is an apocarotenoid known for its role in ROS scavenging and stress signaling, contributing to photoprotection and redox homeostasis (Carnà *et al.*, 2025; Havaux, 2012).

4.2.2. Transcriptomic responses to heavy metal stress in plants

Heavy metal uptake, translocation, and homeostasis are mediated by a variety of metal transporters. Only a few have been well-characterized in plants for both non-essential (Hg, Cd, Cr, Pb, As) and essential (Zn, Mn, Fe) metals. These include the Multidrug and Toxic Compound Extrusion (MATE) proteins, natural resistance-associated macrophage proteins (NRAMPs), CPx-type ATPases, heavy-metal-transporting ATPase (HMAs), cation diffusion facilitators (CDFs), copper transporters, and Zrt-Irt-like (ZIP) family transporters, which act as biological pumps located across cellular and organelle membranes (Rekha *et al.*, 2021). In *Brassica napus*, 270 metal transporter genes (MTGs) were identified and classified into nine superfamilies, including HMA, NRAMP, and sodium phosphate transporters (NPT) (Zhang *et al.*, 2018).

The MATE family consists of secondary active transporters with 12 transmembrane domains and function as H⁺-coupled antiporters across the plasma and tonoplast membranes. These transporters are divided into four groups: the main facilitator superfamily (MFS), the resistance-nodulation-cell division (RND) family, the small multidrug resistance (SMR) family, and the ATP-binding cassette (ABC) superfamily (Paulsen *et al.*, 1996). They are responsible for the extrusion of toxic compounds and metal ions (Upadhyay *et al.*, 2019). Several MATE transporter genes have been reported in *Gossypium raimondii* and *G. arboreum* (Lu *et al.*, 2018). The ATP-binding cassette (ABC) transporters are the largest transporter family in plants. They play important roles in heavy metal detoxification and cellular metal homeostasis (Jalmi *et al.*, 2022). Verrier *et al.* (2008) has divided ABC proteins into 8 categories from ABCA to ABCH. ABC genes have been reported in several plants including *A. thaliana*, *Oryza sativa*, *Hordeum vulgare*, *Prunus mume*, *Solanum lycopersicum*, *Glycine max*, and *Zea mays* (Qiao *et al.*, 2022; Zhang *et al.*, 2020; Hao *et al.*, 2021; Hashmi *et al.*, 2025; Mishra *et al.*, 2019; Aslam *et al.*, 2021; Guo *et al.*, 2022).

Pleiotropic drug resistance (PDR) genes in *Salix purpurea* have been associated with increased tolerance of the plant to HM stress (Chen *et al.*, 2023). Transient silencing of the PDR gene in *Arachis hypogaea* reduced Cd tolerance and increased membrane

permeability indicating its role in metal efflux and ROS scavenging (Wang *et al.*, 2024). Overexpression of genes like ABCC1, PDR8, and phytochelatin synthase in *A. thaliana* improved metal tolerance and sequestration (Leung *et al.*, 2013). Overexpression of high-affinity sulphate transporters enhanced resistance to various abiotic stresses including heavy metals in a limited sulfur environment (Kumar *et al.*, 2019). The findings of Feng *et al.* (2017) shows that YSL gene in *Solanum nigrum* functions as a transporter of metal-nicotianamine complexes under heavy metal stress like that of Cd and Fe. The non-metal transporters like Sugars Will Eventually be Exported transporters (SWEET), phosphate transporters (PHT) and vacuolar amino acid transporters (AVT) also contribute to metal tolerance (Gautam *et al.*, 2022; Wan *et al.*, 2020; Yang *et al.*, 2020).

Cell wall reinforcement acts as the primary defensive mechanism against heavy metal stress. Cell wall functions as a selective barrier against the entry of heavy metal ions by binding and immobilizing them within its structural components such as cellulose, hemicellulose, lignin, pectin and suberin. The thickening or lignification of the cell wall enhances its rigidity and metal-binding capacity that further reinforces the tolerance of plants to heavy metal toxicity. The upregulation of phenylalanine ammonia-lyase (PAL) genes under Cr stress confirmed their role in tolerating Cr toxicity (Yu *et al.*, 2018). Lignin biosynthetic pathway genes like 4 coumarate CoA-ligase (4CL), cinnamyl alcohol dehydrogenase (CAD), coumarate 3-hydroxylase (C3H) and PAL were upregulated under Al stress in rice (Mao *et al.*, 2004). Cd treatment also triggered the genes 4CL, laccase (LAC) and caffeoyl-CoA O-methyltransferase (CAMT) in tomato seedlings (Liang *et al.*, 2022).

Genes such as Xylan O-acetyltransferase (XOAT14), nonspecific phospholipase (NPC4) and pectin acetylesterase (PAE) were found to be upregulated under heavy metal stress in rice (Kuang *et al.*, 2024). Heavy metal stress induced pectin biosynthesis in *Nicotiana tabacum* (Qu *et al.*, 2024) and similar upregulation of PAE genes was observed in *A. thaliana* (Philippe *et al.*, 2017). Cd inhibited the activity of sucrose synthase and sucrose phosphate synthase in maize (Li *et al.*, 2020). Overexpression of GDP-D-mannose 3',5'-epimerase (GME) genes enhanced stress

tolerance in tomato (Zhang *et al.*, 2011). Induction of Polygalacturonase (PG) and Casparian Strip membrane domain proteins under abiotic stresses supports the structural role of cell wall associated genes in stress tolerance (Wang *et al.*, 2020; Liu *et al.*, 2014).

Chelation and sequestration are important defense mechanisms involved in metal detoxification in plants. Genes involved in glutathione metabolism such as glutathione S-transferase (GST), glutathione synthetase (GSS) and glutathione hydrolase (GH) pathways are upregulated during metal stress (Dubey *et al.*, 2010; El-Shabrawi *et al.*, 2010; Yadav *et al.*, 2008). The serine acetyltransferase (SAT), ATP sulfurylase, phytochelatins (PCs) and metallothioneins (MTs) genes work together to produce cysteine derived chelators that bind and detoxify metals alleviate heavy metal stress (Wang *et al.*, 2024; Anjum *et al.*, 2015; Zare *et al.*, 2023, Sharma *et al.*, 2016). Transcriptome analyses in *Miscanthus sinensis* revealed many DEGs linked to photosynthesis, chelation and metal transport (Nie *et al.*, 2021). ABC transporters and glutathione metabolism were identified as important mechanisms for Cr tolerance. A study on transcriptome profiling of *B. napus* cultivars under Cr toxicity has revealed its alleviation by reduced glutathione (GSH) (Gill *et al.*, 2016). Increased expression of PCs and MTs expression was also observed in *Leucaena leucocephala* under Cr stress that reinforces chelation as an important detoxification mechanism (Srivastava *et al.*, 2021). In *Zea mays* inoculated with arbuscular mycorrhizal fungi under Cr(VI) stress, chelation occurred through increased production of glutathione and phytochelatins (de los Angeles Beltrán-Nambo *et al.*, 2021). Also, Cr(VI) was reduced to Cr(III) within root cells that lowered the metal toxicity. Similar upregulation of chelators like organic acid and antioxidant enzymes, along with reduction of Cr(VI) to Cr(III) in root tissues was observed in *B. napus* (Zulfiqar *et al.*, 2023). Cr(VI) to Cr(III) reduction by the enzyme NAD(P)H-dependent dehydrogenase FQR1 was reported in *Callitriche cophocarpa* (Kaszycki *et al.*, 2018).

Flavonoid biosynthesis plays a major role in ROS detoxification. Flavonoid-related genes including PAL, 4CL, cinnamate 4-hydroxylase (C4H), chalcone synthase

(CHS), flavonone 3-hydroxylase (F3H), and anthocyanidin synthase (ANS) are upregulated under Cd, Pb, and Cr stress, contributing to antioxidant defence and metal chelation (Aluko *et al.*, 2025; Parveen *et al.*, 2025). Transcription factors (TFs) such as MYB, NAC, WRKY, AP2/ERF, bHLH, C2H2-Zn finger, and HSFs regulate ROS scavenging and detoxification pathways (Agarwal *et al.*, 2022; Zhang and Lu, 2024).

Mitogen-activated protein kinase (MAPK) signalling is another critical pathway activated under metal stress. The MAPKKK-MAPKK-MAPK cascade phosphorylates TFs like WRKY, MYC2, and ERF1, coordinating defence gene expression and hormone interactions (Mondal, 2023). A 16-fold upregulation of MAPK genes was observed under Ni and Pb exposure in tomato (Can *et al.*, 2025).

Heavy metal exposure alters phytohormone biosynthesis and signalling. In rice under Cr stress, AUX1 and ARF were downregulated, suppressing growth, while IAMT1 was upregulated, promoting auxin methylation and secondary metabolism (Cong *et al.*, 2019). Mixed regulation of PP2C and PYR/PYL genes indicated abscisic acid (ABA) pathway modulation. In *A. thaliana*, downregulation of GH3, SAUR, and NPR1 limited cell expansion and SA signalling, while upregulation of EIN3 and ERF enhanced ethylene-mediated responses (Jalmi *et al.*, 2018).

Exogenous application of jasmonic acid (JA) and gibberellic acid (GA3) improved Cd tolerance through reduced oxidative damage and metal uptake (Ahmad *et al.*, 2021). Brassinosteroid (BR) signalling was suppressed under Cr and Cd stress by downregulation of BRI1 and BAK1, while BIN2 was upregulated. However, compensatory activation of BZR1/BZR2 and JA-related genes (e.g., JAZ, MYC2) indicated an adaptive response (Kim *et al.*, 2021).

Heavy metals impair photosynthetic machinery, damaging chloroplast ultrastructure, inhibiting chlorophyll biosynthesis, and accelerating pigment degradation. Transcriptomic studies have shown that metal stress suppresses Rubisco subunit and chlorophyll biosynthesis genes, leading to reduced carbon fixation (Rai *et al.*, 2016). Overexpression of C4-photosynthetic genes such as

phosphoenolpyruvate carboxylase (PEPC), pyruvate, orthophosphate dikinase (PPDK), and NADP-dependent malic enzyme (NADPME) in C3 plants enhanced photosynthesis and stress tolerance (Yadav *et al.*, 2020). Significant downregulation of PSII-related genes like *psbA*, *psbD*, *psbK* and upregulation of PSI and ATP synthase genes suggests compensatory energy regulation under stress (Batool *et al.*, 2025). Similarly, DEGs related to pigment biosynthesis, electron transport and light-harvesting complex (LHC) genes like *Lhca1* and *Lhcb1* were altered showing metal-induced disruptions to the photosynthetic electron chain (Xie *et al.*, 2015).

Although plant responses to heavy metals such as Cd, Pb, and As have been widely studied, Cr remains relatively unexplored especially with regard to its molecular and metabolic regulation. The present study on *A. tenella* addresses this gap by integrating transcriptomic and metabolomic analyses to provide the understanding of how plant regulates metal transporters, chelation, antioxidant systems, phytohormone signaling and photosynthetic machinery under Cr stress. This work provides novel insights into Cr-specific detoxification and tolerance mechanisms by comparative analysis of gene expression with metabolite accumulation. This comprehensive multi-omics approach establishes a molecular framework for understanding chromium tolerance in plants and identifies potential candidate genes for developing Cr-tolerant phytoremediators.

4.3. Materials and methods

4.3.1. Non-targeted metabolomic profiling analysis

Metabolite Extraction

Plants subjected to Cr stress for 15 days were randomly selected, and their leaves were rinsed thrice with distilled water. Fresh leaves were immediately frozen in liquid nitrogen for metabolites and derivation extraction. The frozen plant leaves were ground to a powder, dissolved in 70% methanol and extracted at 4°C overnight. Samples were filtered through a microporous filter (0.22 µm pore size) and metabolic analysis were performed by liquid chromatography and tandem mass spectrometry (LC-MS/MS). Three biological replicates were performed.

LC-MS/MS Analysis

LC-MS/MS analysis was done using Agilent 6530C Q-TOF LC/MS system with HPLC 1260 Infinity II Prime LC series. The separation was done with C-18 reverse phased column (Agilent poroshell 120 EC-C8 μm , 3.0×150 mm) with a mobile phase that consists of Acetonitrile (A) and 0.1 % Ac WATER (B) in a gradient elution as follows.

Solvent Composition

	Channel	Ch. 1 Solv.	Name 1	Used	Percent (%)
1	A	100.0% Methanol V.03		Yes	0.00%
2	B	100.0% Acetonitrile V.03		Yes	10.00%
3	C		0.1% Ac WATER	No	
4	D	100.0% Organic in Aqueous V.03		Yes	90.00%

Timetable

Time (min)	A (%)	B (%)
2.00 min	10.00%	90.00%
6.00 min	20.00%	80.00%
10.00 min	30.00%	70.00%
14.00 min	40.00%	60.00%
20.00 min	50.00%	50.00%

ESI ionization technique was employed in negative mode and the general MS parameters were optimized as Nebulizer: 40 psi; source temperature: 325°C; Nitrogen flow: 10L/min; jet stream sheath temperature: 345°C. Total ion chromatogram was extracted using Agilent Mass Hunter software (version 10).

The data process was performed using mass spectrometry software analyst 1.6.3. Metabolite information was identified by conducting searches in both internal and public databases (MassBank, KNApSACk, HMDB, MoTo DB, and METLIN).

Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) was carried out to identify significantly different metabolite levels ($p < 0.05$). Differentially expressed metabolites (DEMs) were designated as a log2fold change (FC) ≥ 2 and p -value ≤ 0.5 , along with variable importance in projection (VIP) scores > 1 . The functional annotation of identified metabolites was done using the Kyoto Encyclopedia of Genes and genomes (KEGG) database and MetaboAnalyst 6.0 (<https://www.metaboanalyst.ca>) and analysed the relevant metabolic pathways.

4.3.2. Transcriptome analysis

4.3.2.1. RNA extraction, de novo sequencing, and assembly

Total RNA Extraction and Sequencing. Plants subjected to Cr stress for 15 days were randomly selected, and their leaves were rinsed thrice with distilled water. Fresh leaves were immediately frozen in liquid nitrogen and total RNA was extracted using TRIzol® reagent following the manufacturer's instructions (Invitrogen, USA). The quantity and quality of total RNA was determined using a NanoDrop spectrophotometer and Agilent Bioanalyzer system, respectively. RNA purification, reverse transcription, library construction, and sequencing were performed at LifeCell Pvt Ltd. (Chennai, India) using Illumina NovaSeq 6000.

Data QC. The raw data quality was checked using FastQC and MultiQC software (Andrews, 2017; Ewels *et al.*, 2016). The data was checked for base call quality distribution, % bases above Q20, Q30, % GC, and sequencing adapter contamination.

Pre-Processing and rRNA removal. The raw sequence reads were processed to remove adapter sequences and low-quality bases using fastp v0.12.4 (Chen *et al.*, 2018). From the quality trimmed data, rRNA reads were filtered out using bbmap's v38.18 (Bushnell, 2014) bbdduk algorithm.

Transcripts De novo Assembly. The de novo transcriptome was assembled using Trinity v2.8.5 (Haas *et al.*, 2013). Contigs shorter than 200 bp were removed from

further analysis. The assembled transcripts were then clustered using CD-HIT v4.8.1 (Fu *et al.*, 2012) software by merging transcripts with 90% sequence similarity to reduce redundancy. The clustered transcripts were used to predict protein coding sequences using TransDecoder v5.5.0 facilitating the identification of protein-coding regions and enhancing functional annotation of the transcriptome.

4.3.2.2 Gene functional annotation and characterization

Annotation. The protein sequences were annotated against NCBI non-redundant (nr) and Uniref100 protein database using BLASTp module of Diamond v2.0.15 (Buchfink *et al.*, 2015). Transcripts with E-value $\leq 1e-04$ was considered for downstream analysis. Protein family (PFAM) hits for the transcribed proteins were generated using hmmscan v3.3.2 against the pfam database. The transcript annotations were used to refine the best transcript using TransdecoderPredict. The KOG annotations were obtained by submitting the protein sequences to KAAS (KEGG Automatic Annotation Server) (Moriya *et al.*, 2007). The Gene Ontology (GO) annotations were obtained by mapping Uniref100 IDs via the Uniprot ID mapping tool. For transcription factor prediction, the predicted proteins were annotated by homology search against PlantRegMap/PlantTFDB v5.0 (Jin *et al.*, 2017) database using BLASTp. The Cluster of Orthologous Groups (COG) annotations were obtained by submitting the protein sequences to WebMGA (Wu *et al.*, 2011).

Alignment and Expression Profiling. The quality-filtered reads were mapped to the assembled transcriptome using Bowtie2 v2.4.5 (Langmead and Salzberg, 2012) and reads counts per transcript were extracted using featureCounts v2.0.3 (Liao *et al.*, 2014).

Differential Expression Analysis. Differential expression analysis was carried out using the DESeq2 v1.34.0 package (Love *et al.*, 2014). Transcripts with less than 5 reads in any of the samples were removed from further analysis. The read counts were normalized (variance stabilized normalized counts) using DESeq2 and differential expression analysis was performed. Transcripts with absolute log2 fold

change ($|\log_2FC| \geq 1$ and $p\text{-value} \leq 0.05$) were considered significant. Expression patterns of differentially regulated transcripts were visualized using volcano and MA plots to highlight key differences across samples.

Gene Ontology and KEGG Pathway Enrichment Analysis. The protein IDs with respect to each transcript that showed significant differential expression were used for GO and KEGG pathway enrichment analysis. Enrichment analysis for GO (Biological process, Molecular function, Cellular component) and KEGG Pathway was performed using the ClusterProfiler v4.2.2 (Yu *et al.*, 2012). The clusterProfiler package provides an enricher (enrichment analyser) function for hypergeometric tests. GO terms and KEGG Pathways with adjusted $p\text{-value} \leq 0.05$ are considered significant.

4.3.3. Tissue level Expression analysis of candidate genes involved in metal detoxification and RNA-Seq validation using quantitative RT-PCR

DEGs associated with metal detoxification were identified through comparative transcriptome analysis of control and Cr-treated leaf tissues using DESeq2. Differentially regulated genes in the Cr-treated leaves were analysed for their roles in Cr uptake, transport, and accumulation, metal tolerance, stress responses and adaptations. 8 top candidate DEGs involved in Cr detoxification is randomly selected from *A. tenella* revealed by transcriptome sequencing for subsequent qRT-PCR analysis. Total RNA was extracted from roots, stem and leaves on 0d, 5d, 10d, and 15d for tissue-specific expression analysis using Qiagen RNeasy Plant Mini Kit. The extracted RNA was reverse transcribed into cDNA by HiCapacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The cDNA was used as templates for gene expression pattern tests in qRT-PCR.

Gene-specific primers were designed using IDT PrimerQuest™ (Table 4.1). qRT-PCR assay was performed on a Real Time PCR (Bio-Rad CFX96™ Touch) System in a final volume of 20 μ l containing 2 μ l of a 1/10 diluted cDNA template, 10 μ l of the 2 $\times$ iQ™ SYBR® Green Master Mix (BioRad Laboratories, Inc., USA) and 1 μ l (5 mM) of gene-specific forward and reverse primers. β -actin served as the reference

gene. The qRT-PCR reactions were performed by using the following cycle conditions: an initial 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, and 55°C for 60 s. After obtaining the Ct-value for each reaction, the relative expression was calculated by 2^{-delta Ct} method, with triplicate reactions per sample.

Table 4.1. qRT-PCR primers for selected genes

Gene ID	Gene	Forward primer	Reverse primer
TRINITY_DN3462_c0_g2_i1	HIPP39	AGATGAGCCCAATAAAGGTCAA	ACGCTTTCTGGATGGTAGTG
TRINITY_DN15703_c0_g1_i5	ABCC2	ATCATGGAGTCGCCTTGCAG	GAACGCCCTTGGAGGAACAT
TRINITY_DN6736_c0_g1_i5	CASP	GATTCGCGGCCATAACACTA	AGCGCCACCATCATCAA
TRINITY_DN11388_c0_g1_i1	GST	AAGATGTTTGGAGAGGGATAGTG	GTTGTGCTTTACTTTGGCTTGA
TRINITY_DN7307_c0_g1_i3	XTH32	CCCGTTCCTGCTTCATACA	GAAGAACTCGATCCGCAAGAA
TRINITY_DN49337_c0_g1_i1	PCS3	GGATGTGCTCTCAGTTGTCTT	CAGCCTTCCATGTTCCTCTT
TRINITY_DN8194_c0_g1_i5	YSL1	CTCTCGTGGGGACTGATGTG	TAGCCCTGCACCTAAAAGCC
TRINITY_DN53751_c0_g1_i2	LAC6	AGGACCTCGTGAGCCTAGAG	ATGCGGCTTGTGGACTGTAA

4.4. Results

4.4.1. Metabolomic analysis of *A. tenella* leaves under Cr stress

To understand how the leaves of *A. tenella* respond to Cr stress, untargeted metabolomic (LC-MS/MS) analyses were conducted to identify the different plant metabolites associated with Cr exposure. Multivariate PCA and PLS-DA analyses were initially applied to examine the clustering patterns of the two treatment groups. The results showed a clear separation between the control and Cr-treated samples (Fig. 4.1a, b) indicating distinct metabolite differences. The significant separation within and between groups also shows the good sample consistency and supports the reliability of the metabolite data.

Using the LC-MS/MS platform and available metabolite databases, a total of 74 differential metabolites were identified. The metabolites that changed in abundance under Cr treatment and their overlapping Venn diagram is shown in Fig. 4.2. The types and quantities of metabolites affected by Cr stress in *A. tenella* were also analysed (Fig. 4.3). In general, metabolites such as lipids, organic acids and flavonoids showed the most marked changes. The effects of Cr stress were also

visualized through cluster heat maps and VIP plots that illustrate the trends and expression patterns of the differential metabolites in each group (Fig 4.4, 4.5).

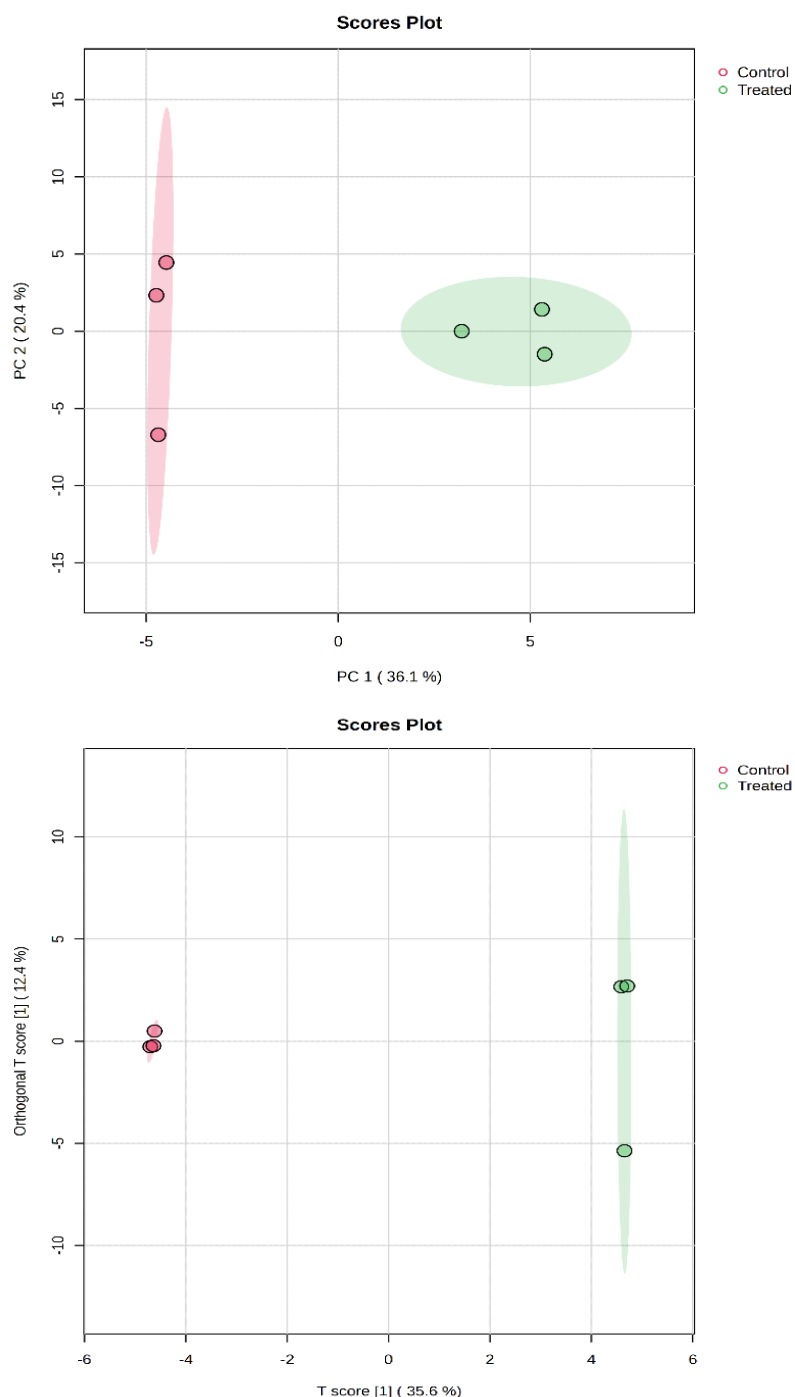


Fig. 4.1. a) PCA score plot, b) OPLS-DA score plot of *A. tenella* leaf metabolites under Cr treatment. X axis represents the first principal component and Y axis represents the second principal component.

Among the 74 differentially abundant metabolites (DAMs) detected, 26 and 43 were significantly upregulated (36%) and downregulated (61%) respectively (Fig. 4.6). This study also identified the top 10 DAMs based on changes in differential metabolite expression (Fig. 4.7). KEGG enrichment pathway analysis was used to identify the major metabolic pathways involved in the plant's response to Cr stress (Fig. 4.8).

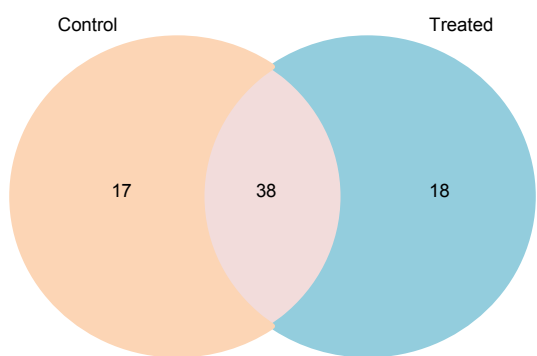


Fig. 4.2. Venn diagram of the number of DAMs in groups. The overlap represents the number of metabolites common to each group and the non-overlap represents the number of metabolites unique to the group.

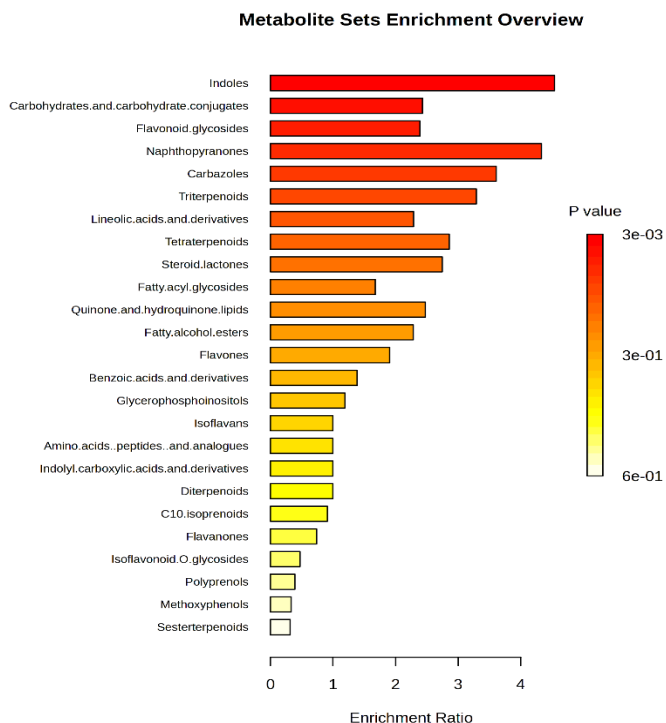


Fig. 4.3. Enrichment analysis showing significantly altered metabolite classes in treated vs control group.

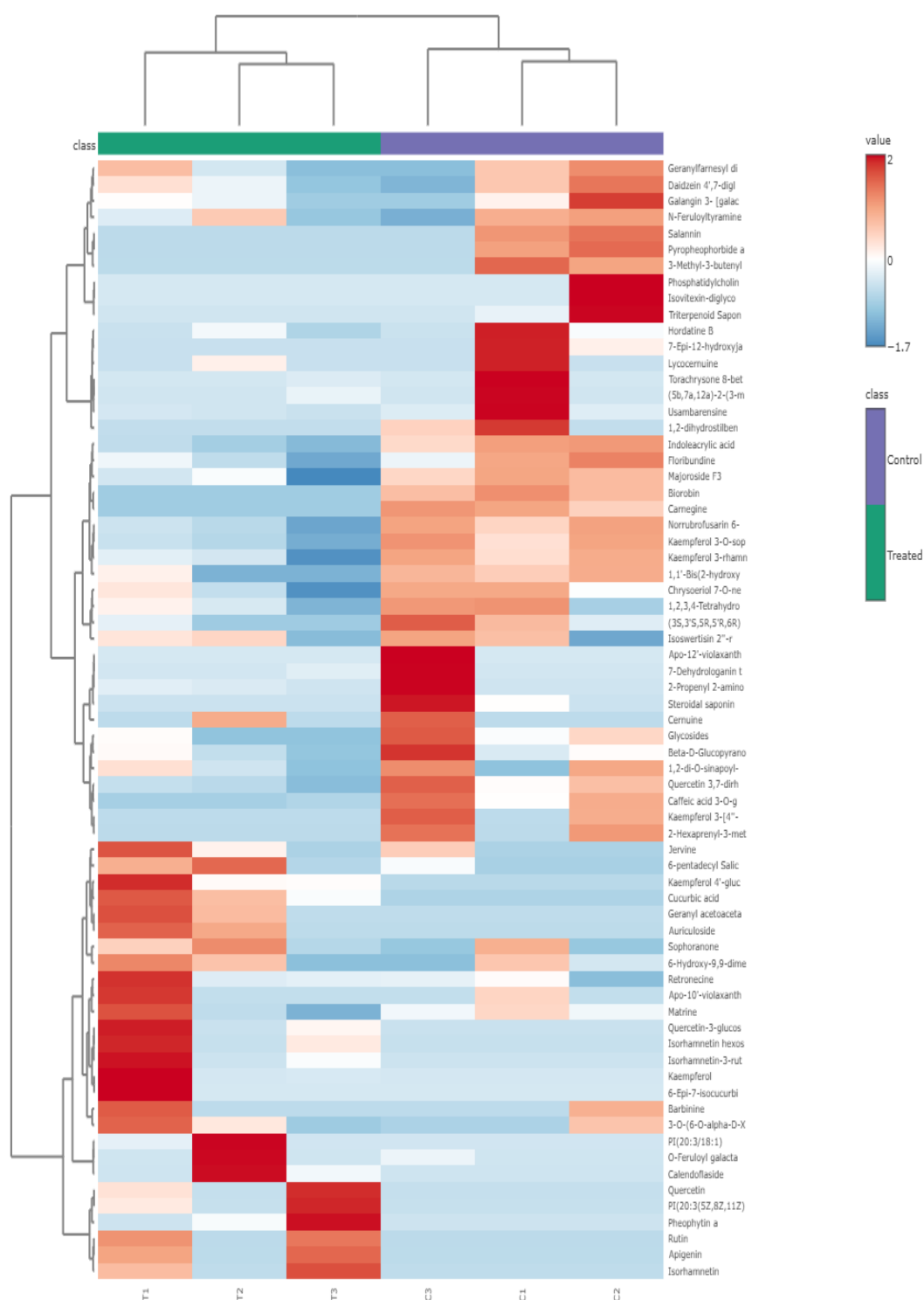


Fig. 4.4. Heatmap of hierarchical clustering of differential metabolites in control and treated samples (n=3 each). Colours represent log₂ fold change values, ranging from blue (low) to red (high).

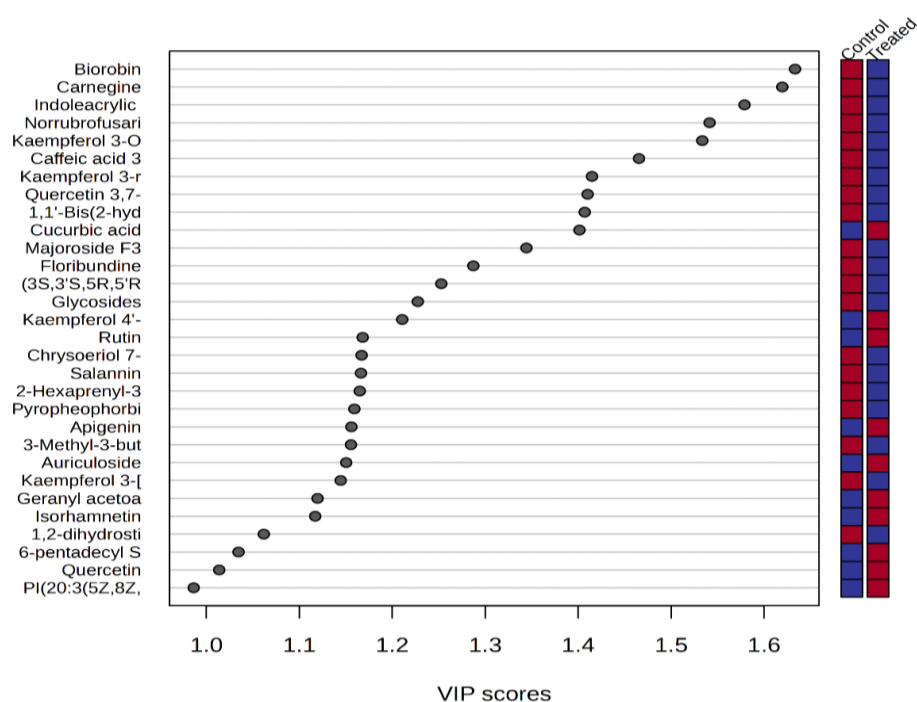


Fig. 4.5. VIP plot of differential metabolites based on PLS-DA analysis. Metabolites with $VIP > 1$ are the main contribute to group separation between control and treated samples.

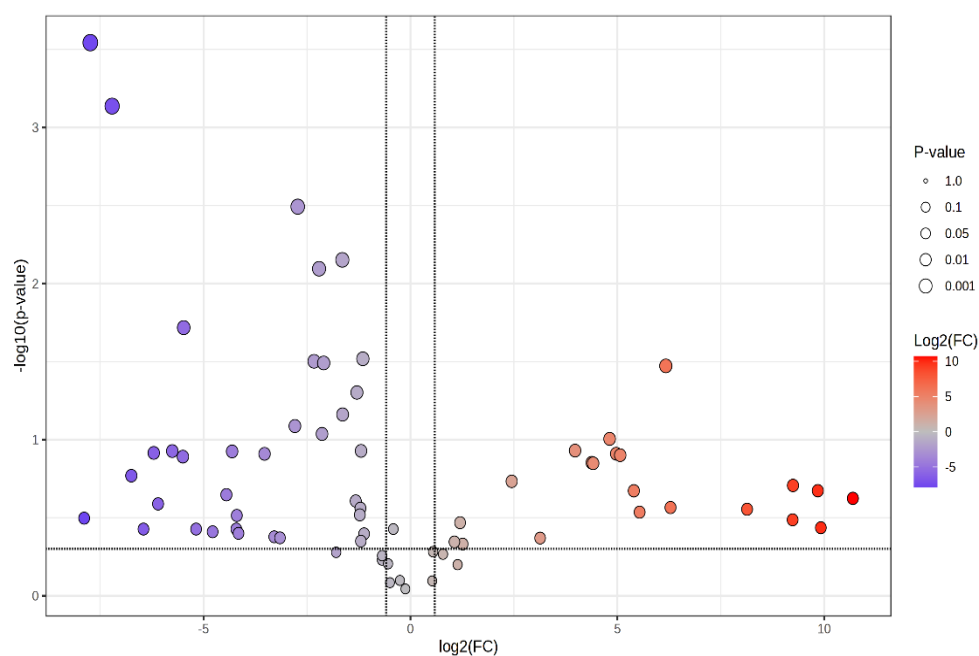


Fig. 4.6. Volcano plots showing differentially abundant metabolites based on $|\log_2FC| \geq 2$ and $p\text{-value} < 0.05$. Red points represent significantly upregulated, and blue represents significantly downregulated metabolites.

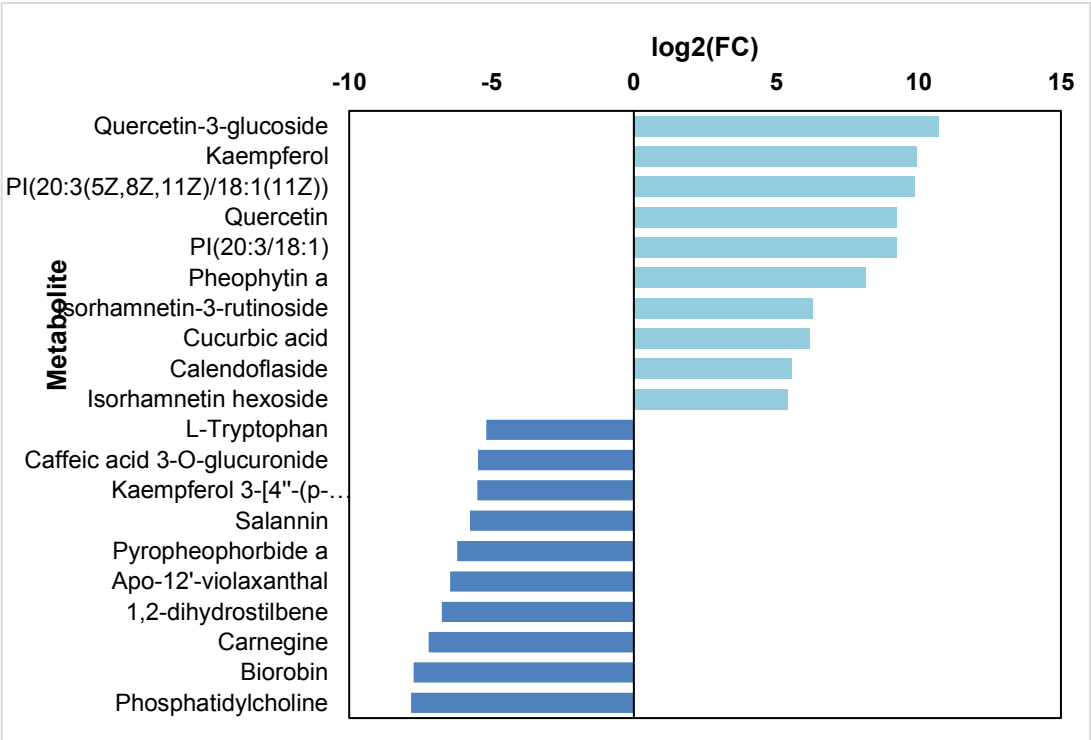


Fig. 4.7. Graph showing the top 10 upregulated and downregulated DAMs identified between control and treated groups based on log₂FC.

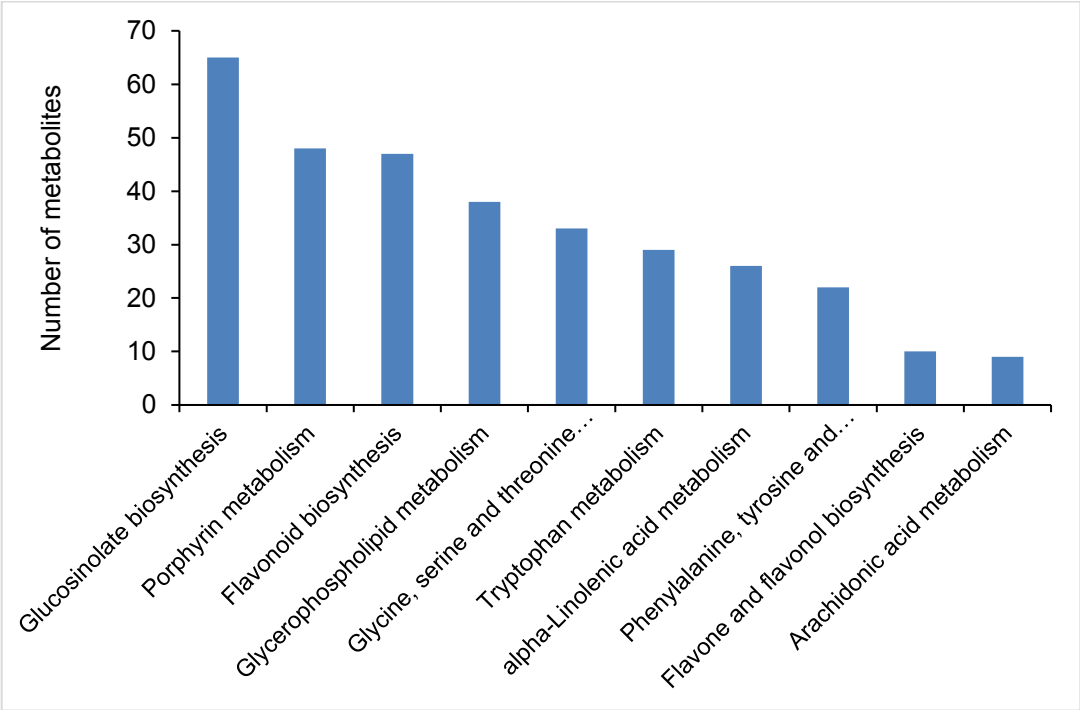


Fig. 4.8. Graph showing the top 10 KEGG functional pathways for metabolite enrichment.

According to the chemical structure, metabolites from the leaf extracts of *A. tenella* were divided into eight major groups (Fig. 4.9), including 8 organic acids, 9 amines, 7 lipids, and 18 flavonoids.

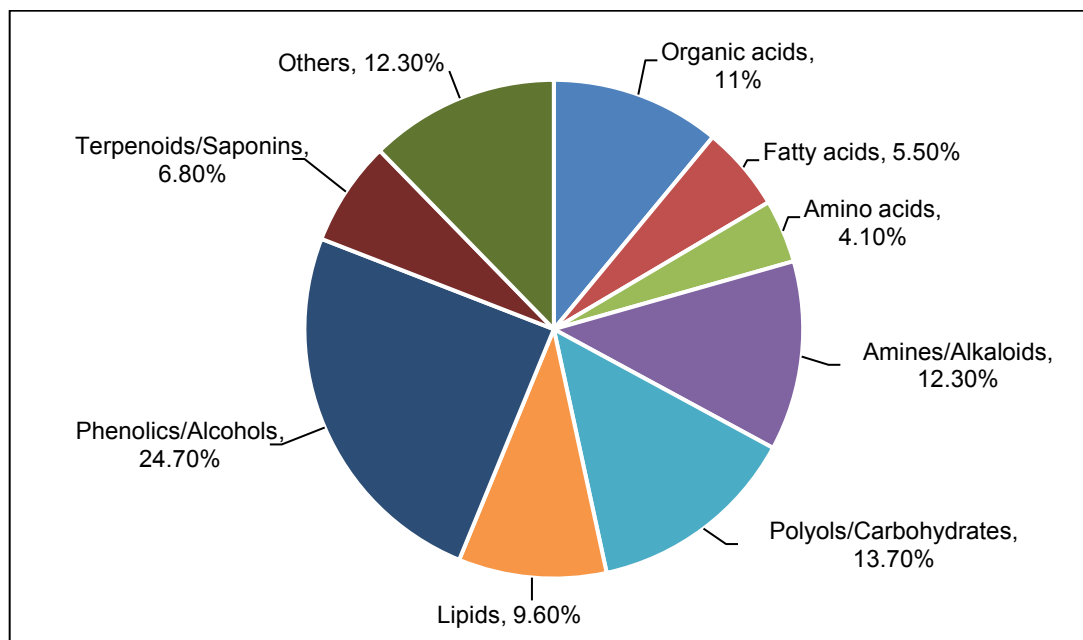


Fig. 4.9. Classification of leaf metabolites of *A. tenella* into major groups based on metabolomic profiling.

Untargeted metabolomic profiling of *A. tenella* leaves exposed to Cr stress revealed distinct reprogramming of secondary metabolism, with major shifts in lipids, organic acids, phenolics, flavonoids, terpenoids, and chlorophyll derivatives. Twenty-six metabolites were upregulated and 43 were downregulated under Cr stress compared to control leaves. Both upregulation and downregulation trends were evident in metabolic responses based on log₂fold changes (log₂FC).

Phenolic compounds especially flavonoids were the mostly upregulated class of metabolites. Quercetin (9.24) and its derivatives like quercetin-3-glucoside (10.69) and rutin (3.98) showed high induction under Cr stress. Other glycosylated flavonoids such as isorhamnetin-hexoside (5.39), isorhamnetin-3-rutinoside (6.28), kaempferol (9.91), and kaempferol 4'-glucoside 7-rhamnoside (4.81) were also highly elevated. A strong upregulation was observed for apigenin (4.97), auriculoid (5.07), calendoflaside (5.54), and 6-epi-7-isocucurbitic acid (4.88). However, quercetin 3,7-dirhamnoside (-2.33), quercetin-3-arabinoside (-2), biorobin

(-7.74), 1,2-dihydrostilbene (-6.74), and caffeic acid 3-O-glucoronide (-5.48) were suppressed. The flavonoid glycosylation was strongly favored in *A. tenella* under Cr exposure, suggesting redirection of carbon flux into antioxidant and ROS scavenging metabolites. Most alkaloids were strongly downregulated under Cr stress, including carnegine (-7.74), usambarensine (-4.2), and lycocernuine (-1.8). This reflects the suppression of nitrogen-rich secondary metabolism, possibly conserving nitrogen for stress-related protein synthesis. Some alkaloids like sophoranone (1.06) and retronecine (1.19) were slightly upregulated which suggests the selective retention of some bioactive compounds.

Saponins and terpenoid derivatives were consistently reduced. Triterpenoid saponins (-7.88), steroidal saponins (-6.10), 2-Hexaprenyl-3-methyl-6-methoxy-1,4 benzoquinone (-4.31) and limonoids like salannin (-5.75) were strongly downregulated. A terpenoid precursor, geranyl acetoacetate (4.38) was upregulated. Some terpenoid glycosides showed moderate positive regulation, but most of them were decreased. This pattern suggests a shift away from constitutive chemical defence metabolites toward stress mitigation pathways. Cell wall-bound phenolics and amines like N-feruloyltyramine showed downregulation. Certain carbohydrate conjugates, such as mannopyranose derivatives () and xylose-linked saccharides () were slightly increased. This can be possibly linked to stress-induced remodeling of hemicellulose and cell wall polysaccharides.

Pigment metabolism was highly affected in *A. tenella* under Cr stress. Photosynthetic intermediates like pyropheophorbide a (-6.2), and other porphyrin intermediates were significantly declined, while pheophytin A (8.14) showed high upregulation. Carotenoid derivatives like Apo-12'-violaxanthal (-6.45) were also downregulated. These reductions were consistent with chlorophyll degradation and suppressed photosynthetic pigment turnover under Cr toxicity. Lipid-derived metabolites like phosphatidylcholine (-7.83) and 7-Epi-12-hydroxyjasmonic acid glucoside (-4.44) were highly downregulated, while phosphatidylinositol derivatives PI(20:3/18:1) (9.23) and PI(20:3(5Z,8Z,11Z)/18:1(11Z)) (9.84) were upregulated. 6-pentadecyl salicylic acid (2.44) was also increased under Cr stress.

Anthraquinone glycosides such as torachryson 8- β -gentiobioside (-4.78) was suppressed. Several metabolites including chrysoeriol 7-O-neohesperidoside (-1.19),

indoleacrylic acid (-2.72), isovitexin-diglycoside (-4.21), L-tryptophan (-5.18), and proline betaine (-3.21) were downregulated. These results together suggest the diversion of resources in *A. tenella* toward flavonoid-mediated antioxidant defence and away from nitrogen-rich alkaloids, energy intensive saponins, and pigment metabolism during Cr detoxification.

4.4.2. Transcriptome analysis of *A. tenella* leaves under Cr stress

Leaf samples from both Cr-treated (T) and control (C) plants were used for total RNA extraction, followed by RNA-seq analysis. To study the response of *A. tenella* to Cr stress, a cDNA library was constructed using RNA from control and Cr-treated leaves. Sequencing on the Illumina Novaseq 6000 platform generated approximately 118 and 123 million paired end reads (raw reads) from control and treated samples respectively (Table 4.2). Raw reads containing adapters, poly-N sequences or low-quality bases were removed to obtain clean reads which were used for all downstream analyses. All raw sequencing data were submitted to NCBI and assigned accession numbers (PRJNA1233897). The high-quality reads were then de novo assembled using Trinity software. A total of 594917 transcripts and 63085 unigenes were identified. Detailed results of transcriptome assembly and functional annotation are shown in Table 4.3.

Table 4.2. Total read number and quality of the raw and clean data

sample	Raw reads	Clean reads	Clean bases	Q30 (%)	GC (%)
C1	38424742	35259450	5.8	92.81	41.88
C2	37830098	35377916	5.71	93.76	43.95
C3	42546178	41590834	6.42	92.05	44.44
T1	40444036	37906952	6.11	93.33	44.39
T2	43375926	38730900	6.55	92.58	40.63
T3	42181218	41442610	6.37	91.62	41.92

In total, 241 million paired end reads containing 36.96 gigabases of raw sequence data were obtained from both samples. The ratio of Q20 (base error rate of less than 1%) reached 93%. The average double-end sequencing fragment was 200 bp in

length. On average, the N50s and N75s of transcripts were 1015 bp and 489 respectively. For the transcripts, 18.75% were longer than 1000 bp, 0.3% were longer than 5000 bp, and 0.02% were longer than 10000 bp (Table 4.3). Sequence alignments with NCBI NR, Uniref100, pfam, TF, GO, COG, and KEGG databases showed 218,631 annotated transcripts (36.75% of all the transcripts) (Table 4.4). The majority of unigenes showed highest similarity to *Chenopodium quinoa*, *Spinacia oleraceae*, and *Beta vulgaris* (Fig. 4.10).

Table 4.3. Summary of assembled transcriptome

Parameter	Annotated transcripts	CD-HIT 90% Clusters
# transcripts (≥ 0 bp)	594917	414323
# transcripts (≥ 1000 bp)	111544	54563
# transcripts (≥ 5000 bp)	1889	812
# transcripts (≥ 10000 bp)	99	44
Largest transcript	30186	30186
Assembled transcriptome size	424999088	252420608
GC (%)	37.15	36.63
N50	1015	755
N75	489	408
Total trinity 'genes'	355564	344281
Total trinity transcripts	594917	414323
Protein coding transcripts	NA	68617
Annotated transcripts	NA	63629 (92.73%)

Table 4.4. Distribution of annotated transcripts

Database	Annotated transcripts
NCBI NR	59155
Uniref100	59195
pfam	55331
KEGG	5721
GO	16475
COG	22562
TF	192

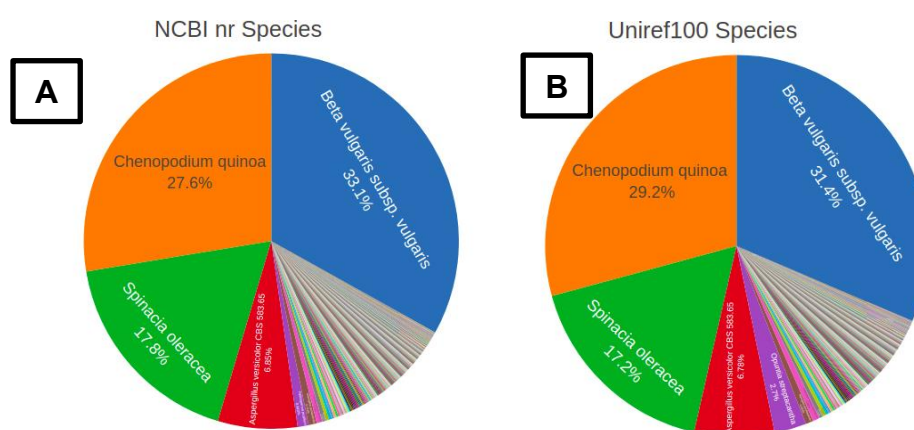


Fig. 4.10. Species distribution of annotated DEGs under chromium stress in *A. tenella*. Graphs represent the top-hit species in databases: A. NCBI nr, B. UniRef100

Differentially expressed genes

DEGs were determined at a threshold of $|\log_2 \text{Fold Change}| \geq 2$ with P value ≤ 0.05 . A total of 47334 genes showed differential expression in the Cr-treated samples compared to control of which, 9900 DEGs were up-regulated and 12,259 DEGs were down-regulated under Cr stress (Fig. 4.11, 4.12a, b). Out of the 1816 significantly expressed unigenes, 1035 were over expressed and 781 were under expressed.

Among 40 DEGs with larger expression differences, 8 genes were for stress response (ACO1, HSFB3, LOX2, PPO, GA3ox1, RPA70B, SCL30, ZAT10), 5 for heavy metal tolerance and detoxification (such as CXE1, CYP72A219, PPO, AASS, PDR1), 6 for stress signalling (such as ACO1, HSFB3, GA3ox1, PP2C25, SCL30, ZAT10), 5 genes for pathogenesis-related and defence (GDSSL1, PPO, SWEET11, PUB22, PDR1), 2 genes for transport and nutrient metabolism (MPC3, UPS2) and 14 uncharacterized or hypothetical proteins.

Gene Ontology (GO) analysis of DEGs in response to Cr in *A. tenella*

The GO functional enrichment analysis showed that 9156 DEGs were involved in biological processes, 8845 in cellular component, and 13335 in molecular function (some transcripts overlapped among the three major categories) (Fig. 4.13).

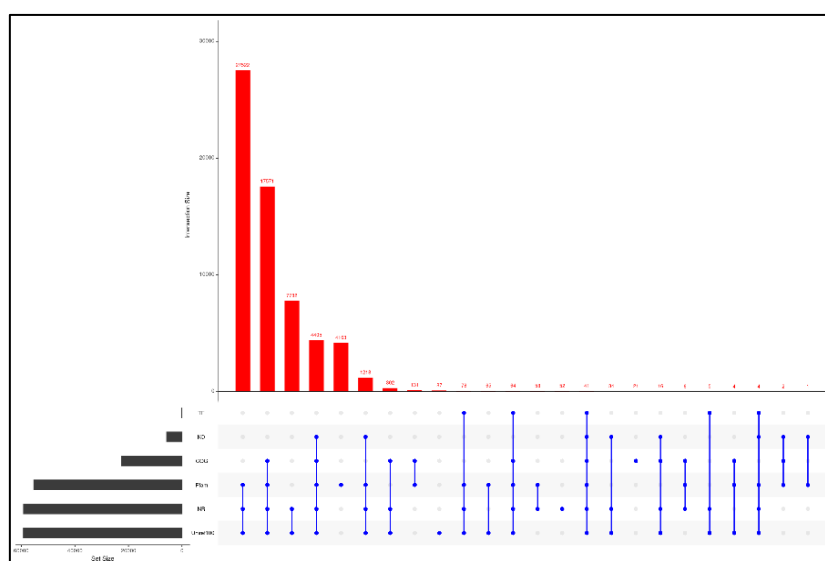


Fig. 4.11. UpSet plot showing the intersection of DEGs identified in the control vs treatment transcriptomic analysis.

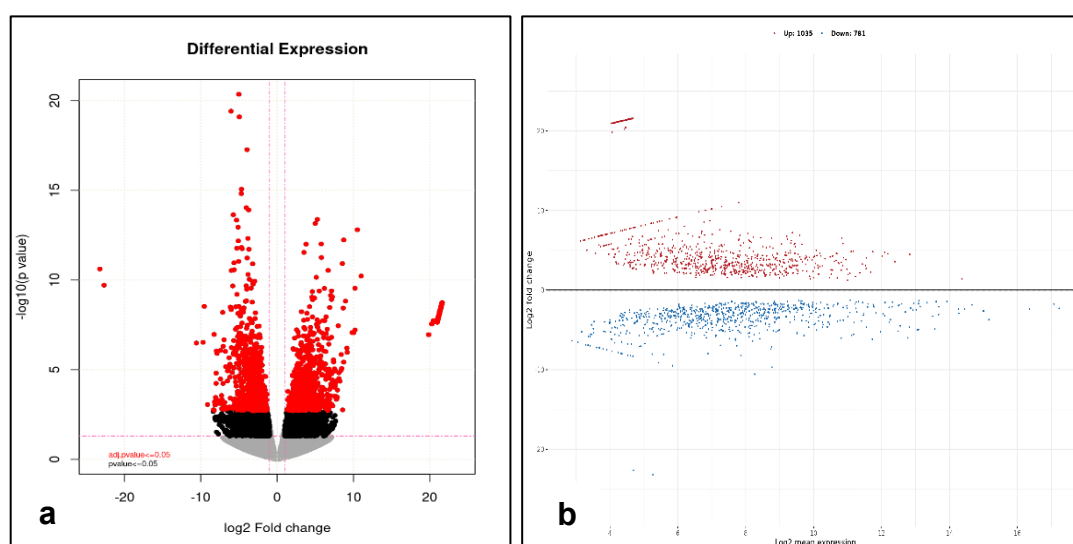


Fig. 4.12. (a) Volcano plot in *A. tenella* leaves exposed to Cr treatments. The dashed line in each volcano plot represents the threshold for an adjusted P- value < 0.05 . Red dots indicate upregulated DEGs, black dots indicate downregulated DEGs, and gray dots represent filtered unigenes that were not significantly regulated. (b) MA plot showing differential gene expression in *A. tenella* leaves exposed to Cr treatment. Upregulated genes are shown in red and downregulated genes in blue, based on $\log_2 \text{FC}$ values.

Based on GO annotation analysis, Cr-regulated DEGs were characterized by an enrichment in protein phosphorylation (2595 DEGs) under biological processes, followed by regulation of DNA-templated transcription (799 DEGs) and defence

response (727 DEGs). As for cellular components, the enrichment was mainly observed in membrane (8473 DEGs) and nucleus (4731 DEGs), and cytoplasm and plasma membrane (1197 DEGs). Within molecular function, the maximum enriched gene categories included ATP binding (5190 DEGs), metal ion binding (2930 DEGs), DNA binding (2036 DEGs), and RNA binding (1626 DEGs).

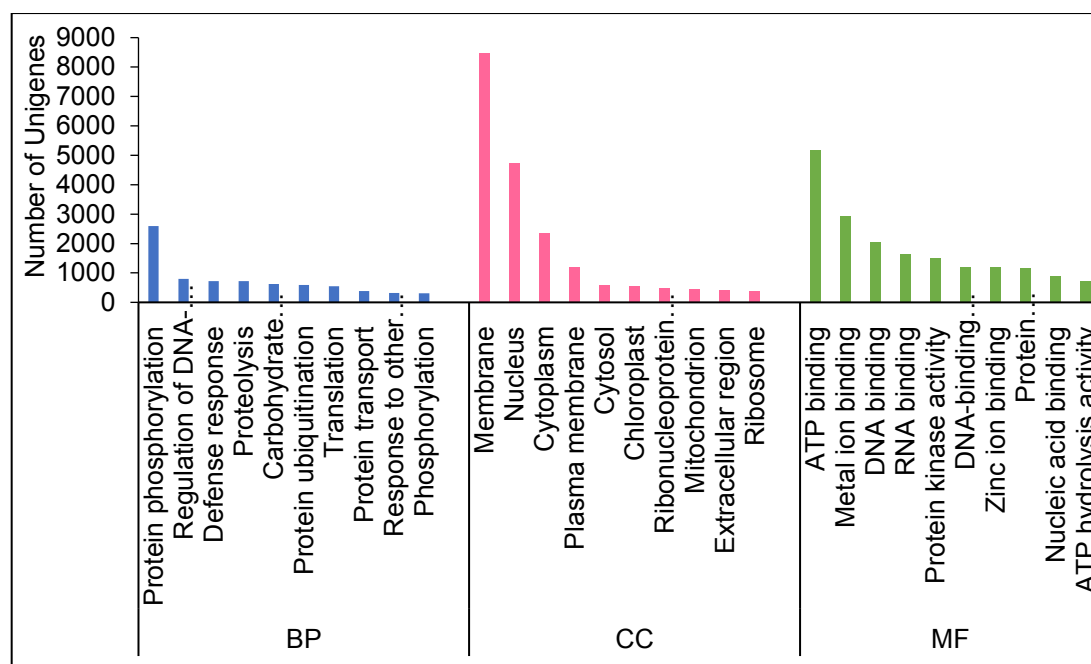


Fig. 4.13. Graph showing GO classification of DEGs in Cr-treated *A. tenella* leaves across biological process, cellular components and molecular function categories.

KEGG enrichment analysis of DEGs

KEGG analysis (Fig. 4.14) showed that under Cr stress, a total of 11.07% of all DEGs were addressed in the 388 pathways. Of them, the protein modification pathway was enriched the most with 504 DEGs, followed by protein ubiquitination (383 DEGs). Amino acid synthesis and metabolism-related pathways, like phenylpropanoid metabolism, cysteine and methionine metabolism, arginine and proline metabolism, phenylalanine/tryptophan biosynthesis contained 14, 32, 29, and 26 DEGs, respectively. Nucleic acid-related pathways, such as pyrimidine metabolism and purine metabolism, contained 45 and 56 DEGs, respectively. Fatty acid biosynthesis, photosynthesis, and glutathione metabolism contained 4, 6, and 7 DEGs, respectively. Respiration-related pathways, like glycolysis/gluconeogenesis,

oxidative phosphorylation, pentose phosphate pathway and glyoxylate and metabolism, contained 83, 2, 40, and 4 DEGs, respectively. Secondary metabolism-related pathways, such as biosynthesis of phenylpropanoid, flavonoid, ubiquinone, porphyrin, and chlorophyll, contained 14, 7, 5, 89, and 27 DEGs, respectively.

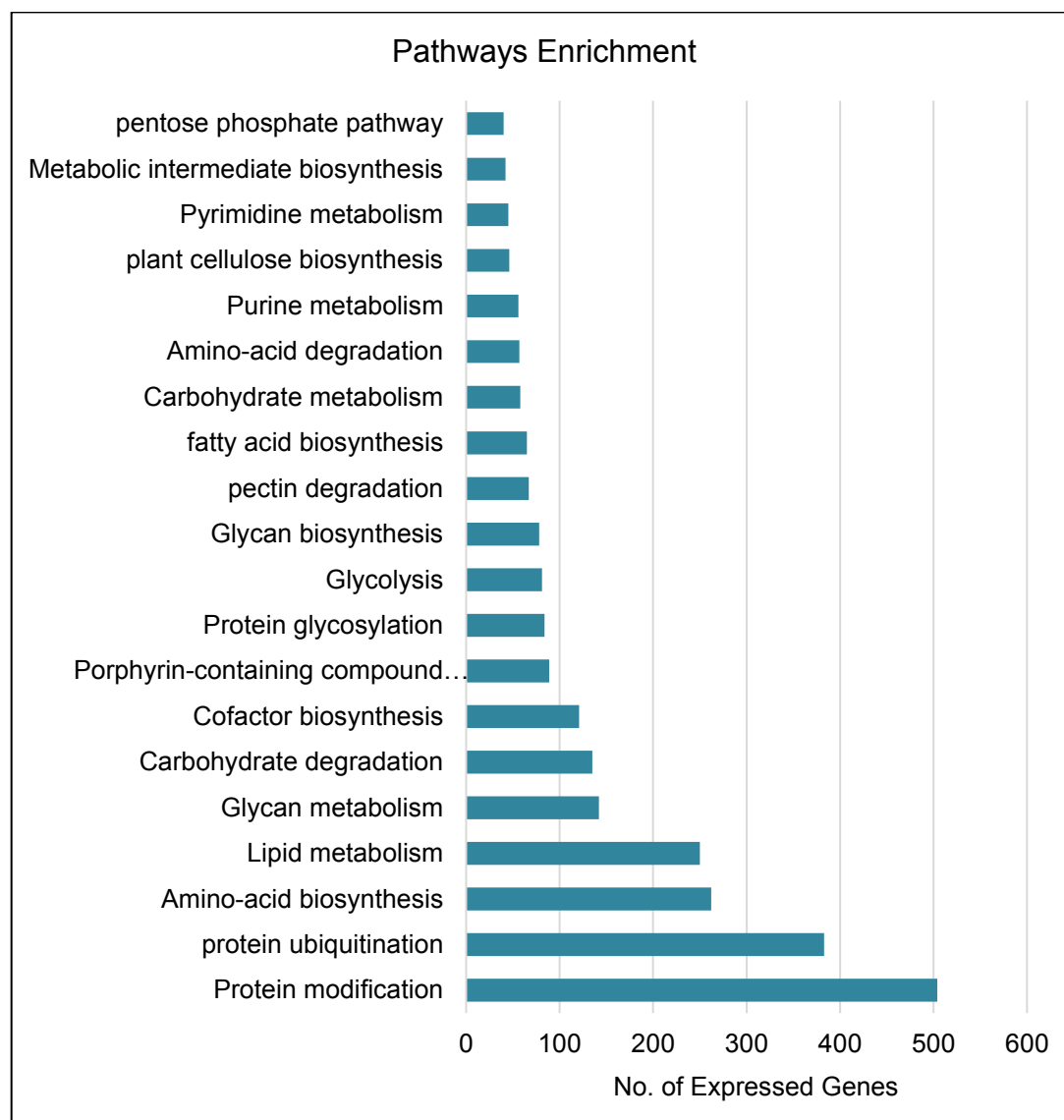


Fig. 4.14. Graph showing KEGG pathway classification of DEGs in Cr-treated *A. tenella* leaves.

Heavy metal transport associated DEGs

As transmembrane metal transporters play important roles in absorption, transport, and detoxification of heavy metals, some important DEGs encoding transporters of

heavy metals were examined (Table 4.5). Several other genes encoding transporters in the DEGs were identified, including 1331 genes in ABC transporter family, 167 MATE family members, 26 genes in ZIP family members, 19 genes in NRT family members, 23 genes in HMA family members, 39 Nramp family members. The expression level of eight ABC transporter genes was remarkably induced in the Cr treatment, including six members of the ABC transporter G family and eight members of the ABC transporter C family. 449 major facilitator superfamily (MFS) DEGs and were also induced by Cr stress. Furthermore, other genes encoding other transporters, such as copper transporter, sugar transporter, phospho-lipid-transporting ATPase, and heavy-metal-associated protein, were notably induced following Cr treatment of *A. tenella*. This suggests that these transporters may play key roles in Cr uptake and transport within the plant as well as maintain the intracellular balance of other ions.

Genes involved in Cr exclusion and transportation were identified such as Pleiotropic drug resistance (PDR). Eight genes encoding PDR were identified including PDR1 and PDR3. In this study, 44 sulfate transporter genes were identified after the Cr stress, including ST 3.5 genes and ST 4.2 genes, which were highly expressed in the leaves. Other transporters in the membrane related to Cr absorption and translocation were also discovered (Table 4.5), such as 19 metal nicotianamine yellow stripe like genes including YSL1, YSL3, YSL6, and YSL7, which were upregulated under Cr stress. Upon Cr treatment, the expression levels of 167 MATE protein genes were significantly upregulated, which may have been associated with Cr efflux. The expression of Plant cadmium resistance 2 (PCR2) was reduced under Cr treatment. In addition, some nonmetallic transporters were differentially regulated under Cr treatment, like bidirectional SWEET transporter, high affinity phosphate transporter (PHT), vacuolar amino acid transporter 1-like (AVT1), and ABC transporter B family member 11-like (ABCB11), which may be involved in the regulation of metal homeostasis in cells.

The expression of ABC transporter genes increased significantly ($p < 0.05$) in the leaves of *A. tenella* after Cr treatment. This suggests an important role for ABC

transporters in the recognizing, binding and eliminating Cr(VI), thereby alleviating the impacts of Cr stress on plants. The expression levels of ABCG36, ABCC14 and ABCF5 were upregulated at 15 days compared with the control group. The translocation of Cr in plants also involves various proteins including plant cadmium resistance proteins (PCRs), metal tolerance proteins (MTPs) and heavy metal-associated isoprenylated plant proteins (HIPPs).

Cell wall components

Cr stress has a significant impact on the expression of cell wall and membrane proteins in *A. tenella*. A total of 435 DEGs ($p < 0.05$) were linked to the cell wall modifications, including cell wall biogenesis, cellulose metabolism, cellulose biosynthesis, pectin metabolism, xylan metabolism, and suberin biosynthesis. Additionally, 1851 DEGs were annotated with pathways related to membrane, transmembrane, and cell wall proteins, indicating a strong response to Cr stress. KEGG analyses revealed one enriched pathway: phenylpropanoid biosynthesis, which plays a key role in cell wall metabolism, particularly lignin biosynthesis. In the study, 233 DEGs (116 upregulated and 117 downregulated) were identified as part of the lignin metabolism, including key enzymes such as phenylalanine ammonia-lyase (PAL), caffeoyl-CoA O-methyltransferase (CAMT), 4 coumarate CoA-ligase (4CL), laccase (LAC), mannitol dehydrogenase (MTDH), and peroxidase (POD). Another 57 DEGs (42 upregulated and 15 downregulated) were involved in the cell wall remodelling, encoding enzymes like pectin acetyltransferase (PAE), UDP-glucosyltransferase (UGT), sucrose synthase (SUS), GDP-mannose 3,5-epimerase (GME), polygalacturonase (PG), and Casparian strip membrane protein (CASPs), suggesting that the cell wall was remodelled in response to Cr stress.

The study also identified 183 DEGs homologous to cell wall metabolism-related genes, with 68 involved in cell wall degradation (34 related to pectin and 34 to cellulose), and another 68 involved in cell wall synthesis (30 in cellulose, 2 in suberin, 7 in lignin, 17 in pectin, and 12 in Casparian strip formation). Additionally, 47 DEGs were involved in cell wall organization, including those encoding expansin proteins and COBRA proteins.

A total of eight GO terms related to the cell wall biosynthesis showed increased gene expression in Cr treated *A. tenella*. KEGG analysis highlighted the enrichment of two key pathways: starch and sucrose metabolism and phenylpropanoid biosynthesis. Genes related to pectin biosynthesis were downregulated, and 22 differentially expressed DEGs encoding plant invertase/pectin methylesterase inhibitor (PMEI) were identified. Wall-associated receptor kinase-like proteins (WAKL) were also significantly upregulated under Cr stress.

Sulphur and glutathione metabolism

Under Cr stress, glutathione (GSH) metabolism in *A. tenella* was significantly affected. A total of 430 DEGs were associated with glutathione S-transferase (GST), glutathione synthetase (GSS), glutathione dehydrogenase (DHAR) and glutathione hydrolase (GH) pathways ($p < 0.05$) indicating that glutathione metabolism play an active role in the plant's response to Cr. In addition, 7 DEGs were identified as iron-sulphur protein-related genes, suggesting a potential link between glutathione metabolism and iron-sulphur proteins in the cellular response. Several DEGs associated with sulphur metabolism were also observably upregulated in response to Cr stress including genes encoding serine acetyltransferase (SAT1, SAT5) and ATP sulfurylase (ATPS). GSH also promotes the production of its derivatives, phytochelatins (PCs) and metallothioneins (MTs) which can chelate metals to form stable complexes. Under Cr stress, the DEGs related to PC and MT biosynthesis in *A. tenella* were significantly ($p < 0.05$) upregulated compared to the control, a pattern that aligns with the qRT-PCR results.

Antioxidant enzyme activity related DEGs

Our experimental results showed that Cr stress led to a significant increase in antioxidant enzyme activities in *A. tenella*, underscoring the critical role of the plant's antioxidant enzyme system in its detoxification mechanisms against Cr stress. Transcriptome analysis revealed 407 DEGs related to antioxidant enzyme activity in the leaves. These included 41 genes encoding superoxide dismutase (SOD), 38 genes encoding catalase (CAT), 133 genes encoding peroxidases (POD),

and 48 genes encoding ascorbate peroxidase (APX). Other enzymes identified in response to Cr stress included respiratory burst oxidase homolog protein H (RBOHH), GST, cytochrome P450 (CYPs), polyphenol oxidase (PPO), and glutathione reductase (GR), and dehydroascorbate reductases (DHAR).

Photosynthesis related DEGs

Under Cr stress, 12 DEGs encoding genes in photosystem II of the light-harvesting pigment complexes (Lhcb1 to Lhcb7) showed significantly reduced expression levels. *A. tenella*, a C3-C4 intermediate plant, exhibits a unique carbon assimilation pathway distinct from typical C3 and C4 plants. The study identified three key enzymes involved in C4 carbon assimilation, with 54 DEGs for phosphoenolpyruvate carboxylase (PEPC), six for pyruvate orthophosphate dikinase (PPDK) and 21 for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The expression levels of PEPC and PPDK increased, while Rubisco expression decreased.

A total of 160 DEGs related to photosynthesis and Cr stress response were found in the transcriptome data. These included DEGs associated with photosystem II (84 DEGs), photosystem I (67 DEGs), the cytochrome b6/f complex (1 DEG), photosynthetic electron transport chain (14 DEGs), and F-type ATP synthase (1 DEG). In PSII, most DEGs including those encoding psbK were downregulated, whereas DEGs related to photosynthetic electron transport were significantly upregulated. DEGs related to PSI showed non-significant changes in expression under Cr stress, while those related to the cytochrome b6/f complex decreased. Within the photosynthetic electron transport chain, one DEG encoding petE, one for petH, five for petF, and three for petL were downregulated under Cr stress. However, four DEGs encoding petF and one encoding petH were upregulated. DEGs related to the light-harvesting chlorophyll II protein complex (LHC), specifically Lhca1 and Lhcb1, were significantly downregulated in response to Cr stress.

Electron transport chain-related DEGs

Among the DEGs related to Cr stress, 159 genes encoding the subunit IV, VA and VIB of cytochrome C oxidase were significantly downregulated, suggesting that Cr inhibited the electron transport chain and disrupted energy synthesis.

Secondary metabolites

KEGG analysis indicated that Cr-induced DEGs were involved in several phenylalanine-related pathways, namely, phenylpropanoid biosynthesis (Ko00940, 89 DEGs), phenylalanine metabolism (Ko00360, 52 DEGs), stilbenoid, diarylheptanoid, and gingerol biosynthesis (Ko00945, 9 DEGs), and flavonoid biosynthesis (Ko00941, 18 DEGs). Additionally, two other phenylalanine-related pathways were identified: “phenylalanine, tyrosine, and tryptophan biosynthesis” (ko00400, 54 DEGs) and “ubiquinone and other terpenoid-quinone biosynthesis” (ko00130, 44 DEGs). Ko00940 plays a central role in the phenylpropanoid metabolism, with phenylalanine synthesized by ko00400 entering pathways ko00360 and ko00130, while the rest enters ko00940, supplying cinnamic acid and cinnamoyl-CoA for downstream pathways ko00941, ko00945, and ko00130. This network, known as phenylpropanoid pathway, begins with the three initial steps catalyzed by phenylalanine ammonium lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumaroyl CoA-Ligase (4CL), which are critical for all subsequent branches and metabolites. Under Cr stress, 33 DEGs were downregulated and 37 upregulated encoding enzymes such as C4H, 4CL, shikimate O-hydroxycinnamoyltransferase (E2.3.1.133, HCT), caffeoyl CoA O-methyl transferase (E2.1.1.104, CCoAOMT), arogenate dehydratase (ADT), and NAD(P)H quinone oxidoreductase 1 (NQO1).

The transcriptome data suggests that *A. tenella* responds to Cr stress by upregulating enzymes involved in flavonoid biosynthesis. Seventeen DEGs in this pathway were upregulated, while ten were downregulated. The downregulated genes included those encoding CCoAOMT, HCT, flavone synthase II (CYP93B2_16), flavanone-3-hydroxylase (F3H), and chalcone synthase (EC:2.3.1.74,

CHS). The upregulated DEGs encoded naringenin 3-dioxygenase (EC: 1.14.11.9), trans-cinnamate 4-monooxygenase (EC:1.14.14.91, CYP73A), flavonoid 3'-monooxygenase (EC:1.14.14.82, CYP75B1), chalcone isomerase (E5.5.1.6, CHI), flavonol synthase (EC:1.14.20.6, FLS), leucoanthocyanidin reductase (LAR), anthocyanidin synthase (EC:1.14.20.4, ANS), CHS, and F3H. These results indicate that flavonoid biosynthesis is highly regulated under Cr treatment.

In the phenylpropanoid biosynthesis pathway, especially in lignin biosynthesis, expression of key genes was reduced by Cr treatment. Enzymes like caffeic acid 3-O-methyltransferase (COMT), plant peroxidase (POX), cinnamyl alcohol dehydrogenase (CAD), and CCoAOMT were downregulated, suggesting that *A. tenella* does not promote lignin biosynthesis as a defence mechanism against Cr stress.

Transcription factors

Transcription factors (TFs) are key regulators of gene expression, playing crucial roles in plant development and adaptation to abiotic stresses. They transmit signals and regulate downstream genes, making them important hubs in gene regulation networks. In this experiment, the expression levels of 2238 DEGs related to TFs were significantly altered in *A. tenella* leaves under Cr(VI) stress. The upregulated TF genes mainly belonged to several families, including WRKY, MYB, NAC, EREBP, AP2/ERF, and zinc finger protein genes. A total of 270 WRKY TF genes were affected by Cr stress, with some, such as WRKY 75, WRKY 21, and WRKY 74, known for their roles in metal tolerance. 222 DEGs were identified as AP2/ERF TFs, most of which were upregulated, with some showing a fold change (log2 value) of up to 6.5. Similar expression patterns were observed in C2H2, MYB, and NAC genes. Several heat shock transcription factors (HSPs) were also upregulated, with 174 DEGs encoding HSP proteins detected, including chloroplastic small heat shock unigenes (sHSPs) involved in metal stress response.

The TCP family was mainly downregulated under Cr stress. The EREBP subfamily TFs, including 27 DEGs were responsive to abiotic stress. The significant

upregulation of RWP-RK domain containing proteins and dehydration-responsive element binding (DREB) proteins like DREB2C, DREB2F indicates the role for AP2/EREBP TFs in Cr stress response. Ten DEGs encoding BES1/BZR1 homolog proteins involved in brassinosteroid signalling were significantly downregulated. Furthermore, Cr stress regulated 378 genes encoding bHLH TFs, 415 encoding C2H2 TFs, and 916 encoding MYB TFs, highlighting their involvement in the plant's response to Cr stress.

α -Linolenic acid metabolism

A total of 51 DEGs were annotated to 7 enzymes involved in α -linolenic acid metabolism. The upregulated enzymes included hydroperoxide dehydrogenase (AOS, EC: 4.2.1.92), OPC-8:0 CoA ligase 1 (OPCL1), acetyl CoA acyltransferase (fadA), while alcohol dehydrogenase class-P (ADH1, EC: 1.1.1.1), jasmonate O-methyltransferase (JMT, EC: 2.1.1.141), enoyl CoA hydrogenation/3-hydroxyacyl CoA dehydrogenase (MFP2), were downregulated. The expression of chloroplastic oxoene reductase (CEQORH) was also downregulated.

Protein kinases

The “protein phosphorylation (GO:0006468)” category showed the highest enrichment for BP, while “protein kinase activity (GO:0004672)” and “protein serine/threonine kinase activity (GO:0004674)” were significantly enriched for MF. In this study, the MAPK signalling cascade activated various kinases and transcription factors, such as WRKY 22 and 33, MYC2 and ERF1, which in turn induce diverse stress and growth responses. A total of 152 transcripts related to the MAPK signalling pathway were upregulated, while 58 were downregulated.

Genes such as MAP kinase (MAPK), WRKY transcription factor 33 and 22 (WRKY33, WRKY 22), aminocyclopropane-1-carboxylate synthase (ACS), mitogen-activated protein kinase kinases 4/5 (EC:2.7.12.2, MKK4/5), serine/ threonine-protein kinase OXI1 (OXI1), mitogen-activated protein kinase kinase 9 (EC:2.7.12.2, MKK9), serine/threonine-protein kinase TOR (TOR), respiratory burst oxidase (RBOH) were upregulated in response to Cr stress. Additionally, four genes

involved in calcium signalling - mitochondrial calcium uniporter (MCU), phosphatidylinositol phospholipase C, delta (EC:3.1.4.11, PLCD), calcium-dependent protein kinase (EC:2.7.11.1, CPK), and voltage-dependent anion channel protein 2 (VDAC2) were also upregulated. The fold change (log₂value) of VDAC2 was as high as 22.

Plant hormones like abscisic acid (ABA), jasmonic acid, and ethylene likely activated the MAPK signaling pathway to enhance the plant adaptability to Cr stress in *A. tenella*. Key genes such as PP2C, MAPKKK 18-like, and CAT 1 were significantly ($p < 0.05$) upregulated in ABA via the MAPK signalling pathway, helping the plant tolerate Cr stress. The significantly upregulated ($p < 0.05$) expression levels of RAN 1 and ERF1 in ethylene-related MAPK signalling and MYC2 in jasmonic acid-related MAPK signalling, indicate enhanced tolerance to Cr-induced stress.

Phytohormone signal transduction

KEGG analysis displayed a total of 154 DEGs were identified between the Cr-treated and control groups in the plant hormone biosynthesis and signal transduction pathway (ko04075). Among these, 54 DEGs were upregulated.

Significant alterations in auxin (IAA) biosynthesis and signal transduction pathways were observed under Cr stress in *A. tenella*. The biosynthesis of auxin through tryptophan (Trp) to indole-3-pyruvic acid (IPA) and IPA to indole-3-acetic acid (IAA) pathway was upregulated in Cr-treated leaves, indicating an attempt by the plant to maintain auxin levels under stress. However, genes associated with auxin signalling including Auxin-resistant 1 (AUX1) and Auxin Response Factor (ARF) were significantly downregulated. Early auxin-responsive gene families like auxin/indole-3-acetic acid (Aux/IAA), GH3, and SAUR also showed a predominant downregulation in Cr-treated leaves, reflecting a suppression of auxin signalling in response to Cr stress. Overall, 33 DEGs were associated with IAA signal transduction, most of which were downregulated under Cr stress.

The study observed a significant upregulation of genes involved in abscisic acid (ABA) biosynthesis in Cr-treated plants. The biosynthetic conversions, from zeathin to xanthoin, xanthoin to abscisic aldehyde, and abscisic aldehyde to ABA, showed high expression levels in treated leaves. This upregulation suggests that ABA plays a key role in the plant's response to Cr stress, likely helping to mediate stress tolerance mechanisms such as stomatal closure which is commonly regulated by ABA during abiotic stress. However, in ABA signalling pathway, the ABA receptor genes (PYR/PYL) showed mixed regulation. Six DEGs encoding the ABA receptor PYR/PYL were identified, of which four were downregulated and two were upregulated. The Protein phosphatase 2C (PP2C) gene, a key regulator in ABA signalling was also upregulated under Cr treatment.

The ethylene biosynthesis pathway showed both up- and down-regulated genes in response to Cr stress. The gene encoding S-adenosyl methionine synthetase (SAM synthetase) which converts methionine to SAM, was upregulated in treated leaves, enhancing the availability of the precursor for ethylene production. However, enzymes involved in the subsequent steps of ethylene biosynthesis 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and ACC oxidase (ACO) were downregulated. This suggests a suppression of ethylene production in response to Cr stress. Despite the reduced biosynthesis, ethylene signalling genes such as ethylene receptor (ETR), ethylene-insensitive 3 (EIN3), and ethylene-responsive factor (ERF) were upregulated, while ethylene-insensitive protein 2 (EIN2) was downregulated. This pattern may imply that the ethylene signalling pathway remains active even with reduced ethylene levels, potentially as a stress adaptation mechanism. A total of seven DEGs were identified to be involved in ETH signal transduction.

In the gibberellin signalling pathway, a key growth repressor protein DELLA was downregulated, whereas phytochrome-interacting factor 3 (PIF3) was upregulated under Cr stress. During GA biosynthesis, the expression of gibberellin 3 β -dioxxygenase (GA3ox; EC:1.14.11.15) which catalyses the formation of GA precursors, was upregulated. Gibberellin receptor (GID1) genes were also

upregulated in Cr-treated leaves, indicating enhanced GA signalling activity. Genes associated with cytokinin biosynthesis and signalling were mostly downregulated in Cr-treated leaves. The adenylate dimethylallyltransferase (IPT; EC:2.5.1.27, 2.5.1.112), involved in cytokinin synthesis, was downregulated. Similarly, genes encoding histidine phosphotransfer proteins (AHP) and two-component response regulators of the ARR-A and ARR-B families were downregulated, suggesting reduced cytokinin signalling activity.

Differential regulation of brassinosteroid-related genes was observed under Cr stress. The brassinosteroid receptor (BRI1) was downregulated, while the negative regulator BIN2 (EC:2.7.11.1) was upregulated, suggesting inhibition of BR signaling. The brassinosteroid-resistant transcription factors (BZR1/2) involved in BR-regulated growth were upregulated. Downregulation was observed for xyloglucan:xyloglucosyl transferase (TCH4; EC:2.4.1.207), a cell wall modifying enzyme, and cyclin D3 (CYCD3), a cell cycle-related gene, both of which are downstream BR-responsive genes. BRI1-Associated receptor kinase 1 (BAK1, EC:2.7.10.1) and BR-signalling kinase (BSK; EC:2.7.11.1) showed differential regulation.

A total of six DEGs were identified as being related to jasmonic acid (JA) signalling pathway under Cr stress. Jasmonic acid-amino synthetase (JAR1; EC:6.3.2.52) and transcription factor MYC2 were upregulated, indicating activation of JA signalling which is commonly involved in stress responses and defence mechanisms. Coronatine-insensitive protein 1 (COI1) and some jasmonate ZIM-domain-containing proteins (JAZ) were downregulated. In the salicylic acid (SA) signalling pathway, genes associated with defence responses like pathogenesis-related protein 1 (PR1) genes were upregulated, while key regulator of SA-mediated defence, non-expressor of pathogenesis-related genes 1 (NPR1) was downregulated. This indicates an enhanced SA response under Cr stress, likely contributing to enhanced pathogen defence. TGA transcription factors were upregulated in Cr-treated leaves.

The combined regulation of these hormone pathways highlights the complex and varied response of plant to Cr stress, involving both stress adaptation and growth regulation mechanisms.

Table 4.5. Significant DEGs and associated metabolic pathways involved in Cr uptake, transport and detoxification

Unigenes ID	Log ₂ FC	Description
Metal transporters		
TRINITY_DN123877_c0_g1_i1	-1.47	ABC transporter A family member 12
TRINITY_DN82698_c0_g1	4.03	ABC transporter B family member 11
TRINITY_DN142585_c0_g1	-2.86	ABC transporter B family member 13
TRINITY_DN211904_c0_g1	4.22	ABC transporter B family member 21
TRINITY_DN173717_c0_g1	-3.04	ABC transporter B family member 9
TRINITY_DN104179_c0_g1_i1	3.31	ABC transporter C family member 10
TRINITY_DN86764_c0_g1	2.40	ABC transporter C family member 10
TRINITY_DN86764_c0_g1	2.15	ABC transporter C family member 10
TRINITY_DN251684_c0_g1	-3.49	ABC transporter C family member 3
TRINITY_DN224291_c0_g1	-5.58	ABC transporter C family member 8
TRINITY_DN6913_c0_g1	2.22	ABC transporter G family member 11
TRINITY_DN274226_c0_g1	-2.94	ABC transporter G family member 20
TRINITY_DN247846_c0_g1	-2.44	ABC transporter G family member 39
TRINITY_DN34549_c1_g1	-9.54	ABC transporter G family member 8
TRINITY_DN53611_c1_g1	2.51	ABC transporter I family member 20
TRINITY_DN16816_c0_g1	2.05	Cadmium/ zinc-transporting ATPase HMA3
TRINITY_DN67025_c0_g2	5.08	Copper-transporting ATPase HMA5
TRINITY_DN91110_c0_g1	3.60	Heavy metal-associated isoprenylated plant protein 30
TRINITY_DN156052_c0_g1	-2.48	Heavy metal-associated isoprenylated plant protein 31
TRINITY_DN3462_c0_g2	3.17	Heavy metal-associated isoprenylated plant protein 39
TRINITY_DN238098_c0_g1	3.42	Heavy metal-associated isoprenylated plant protein 41
TRINITY_DN6130_c0_g1	-1.88	Heavy metal-associated isoprenylated plant protein 7
TRINITY_DN81388_c0_g1	-3.38	Metal transporter Nramp2
TRINITY_DN1582_c4_g1	1.02	Metal transporter Nramp2 isoform X2
TRINITY_DN44200_c0_g1	2.39	Metal transporter Nramp5
TRINITY_DN195133_c0_g1	2.02	Metal-nicotianamine transporter YSL7
TRINITY_DN1690_c0_g1	4.39	Pleiotropic drug resistance protein 1
TRINITY_DN247003_c0_g1	-1.92	Pleiotropic drug resistance protein 1

TRINITY_DN120570_c0_g2	2.92	Pleiotropic drug resistance protein 3
TRINITY_DN273317_c0_g1	-2.46	Pleiotropic drug resistance protein 3
TRINITY_DN68131_c0_g1	5.66	Protein NRT1/ PTR FAMILY 1.2
TRINITY_DN61623_c0_g1	2.04	Protein NRT1/ PTR FAMILY 1.2
TRINITY_DN6225_c0_g1	2.51	Protein NRT1/ PTR FAMILY 2.9
TRINITY_DN13181_c0_g1	3.34	Protein NRT1/ PTR FAMILY 3.1
TRINITY_DN43527_c0_g1	-3.99	Protein NRT1/ PTR FAMILY 5.6
TRINITY_DN234970_c0_g1	2.29	Protein NRT1/ PTR FAMILY 7.3
TRINITY_DN77091_c0_g1	1.17	Zinc transporter 1
TRINITY_DN198_c1_g1	2.13	Zinc transporter 7
Cell wall metabolism		
TRINITY_DN43479_c1_g1	-3.90	4-coumarate--CoA ligase 1
TRINITY_DN139780_c0_g1	4.17	4-coumarate-- CoA ligase 2
TRINITY_DN157469_c0_g1	-2.71	4-coumarate-- CoA ligase 6
TRINITY_DN99619_c0_g1	3.58	4-coumarate-- CoA ligase 7
TRINITY_DN25919_c0_g1	3.04	Caffeic acid 3-O-methyltransferase
TRINITY_DN94021_c0_g1	-4.16	Caffeic acid 3-O-methyltransferase
TRINITY_DN16200_c0_g1	-2.45	Caffeic acid 3-O-methyltransferase 1
TRINITY_DN21282_c0_g1	-1.53	Callose synthase 10
TRINITY_DN6736_c0_g1	-3.00	Casparian strip membrane protein 1E1
TRINITY_DN9945_c0_g2	-4.63	Casparian strip membrane protein 3
TRINITY_DN103005_c0_g1	-2.83	Cellulose synthase
TRINITY_DN140849_c0_g1	-4.61	Cellulose synthase A catalytic subunit 4
TRINITY_DN30206_c0_g1	-2.25	Cellulose synthase A catalytic subunit 6
TRINITY_DN864_c0_g1	2.52	Cellulose synthase-like protein G2
TRINITY_DN48310_c0_g1	-4.14	Cinnamyl alcohol dehydrogenase 6
TRINITY_DN73_c0_g2	-3.74	Expansin-A10
TRINITY_DN2775_c0_g1	-3.33	Expansin-A3
TRINITY_DN73_c0_g1	-5.04	Expansin-A4
TRINITY_DN23312_c0_g1	5.09	Expansin-B1
TRINITY_DN17886_c0_g2	-3.29	Expansin-B3
TRINITY_DN33532_c0_g1	4.67	Laccase-12
TRINITY_DN221769_c0_g1	-4.53	Laccase-17
TRINITY_DN41853_c1_g1	-2.49	Laccase-2
TRINITY_DN41853_c0_g1	-1.94	Laccase-3
TRINITY_DN53751_c0_g1	-3.52	Laccase-6
TRINITY_DN189697_c0_g1	-2.75	Laccase-7

TRINITY_DN74617_c0_g1	1.20	Pectin acetylerase 8 isoform X1
TRINITY_DN211821_c0_g1	-1.76	Pectin acetylerase 9
TRINITY_DN8493_c0_g1	-3.07	Pectin acetylerase 9
TRINITY_DN90862_c0_g1	-3.33	Pectinesterase
TRINITY_DN203567_c0_g1	2.08	Pectinesterase 2
TRINITY_DN45441_c0_g1	-2.93	Pectinesterase 2
TRINITY_DN36810_c0_g1	-2.20	Pectinesterase inhibitor 7-like
TRINITY_DN94146_c0_g1	2.22	Pectinesterase inhibitor 9
TRINITY_DN80723_c0_g2	-6.48	Peroxidase 25
TRINITY_DN86036_c0_g1	-3.45	Peroxidase 47
TRINITY_DN18537_c0_g1	-3.79	Peroxidase 64
TRINITY_DN41088_c0_g1	-5.44	Phenylalanine ammonia-lyase
TRINITY_DN250650_c0_g1	-2.24	Polygalacturonase
TRINITY_DN33557_c0_g1	-1.97	Sucrose synthase 7 isoform X3
TRINITY_DN23923_c0_g1	1.47	UDP-glucose 4-epimerase
TRINITY_DN1890_c1_g1	2.81	Xyloglucan endotransglucosylase/hydrolase protein 30
TRINITY_DN7307_c0_g1	-4.03	Xyloglucan endotransglucosylase/hydrolase protein 32
TRINITY_DN1890_c1_g2	3.17	Xyloglucan endotransglucosylase/hydrolase protein 33
TRINITY_DN1890_c1_g1	-3.66	Xyloglucan endotransglucosylase/ hydrolase protein 8
Glutathione metabolism and metal chelation		
TRINITY_DN284496_c0_g1	3.50	Cysteine synthase A
TRINITY_DN12399_c0_g1	2.37	Cysteine synthase isoform X1
TRINITY_DN12399_c1_g1	-1.32	Cysteine synthase isoform X3
TRINITY_DN275923_c0_g1	1.23	Glutathione gamma- glutamylcysteinyltransferase 1 isoform X1
TRINITY_DN49337_c0_g1	1.52	Glutathione gamma- glutamylcysteinyltransferase 3
TRINITY_DN11388_c0_g1	4.66	Glutathione S-transferase
TRINITY_DN32301_c0_g1	3.69	Glutathione transferase GST 23
TRINITY_DN78793_c0_g1	1.11	Nicotianamine synthase
TRINITY_DN48883_c0_g1	3.89	Serine acetyltransferase 1

4.4.3. Tissue level expression of selected genes involved in Cr detoxification in *A. tenella*

The relative expression levels of eight selected genes were analyzed in leaves, stems, and roots of *A. tenella* under Cr treatment using RT-PCR across four-time intervals (0, 5, 10, and 15 days). Expression patterns revealed distinct tissue- and time- dependent variations and were expressed as fold change (mean $\pm$ SE).

HIPP39

The expression level of HIPP39 in leaf tissues was 1.55 ± 0.35 at 0d. It was slightly downregulated to 1.15 ± 0.11 at 5d. The gene expression increased by to 2.95 ± 0.34 at 10d and 3.31 ± 0.25 at 15d, reflecting an upregulation by 1.9- and 2.1-fold. This gradual upregulation under prolonged exposure of Cr indicates a strong inducible response to metal stress in leaves (Fig. 4.15A).

The expression of HIPP39 in stem tissues showed a consistent increase from 1.18 ± 0.18 on 0d to 3.03 ± 0.33 at 10d, marking a 2.6-fold induction. However, the transcript level declined to 1.41 ± 0.19 at 15d, suggesting a transient upregulation pattern that peaked mid exposure and then stabilized (Fig. 4.15A).

In root tissues, the HIPP39 expression showed an increase of 1.2-fold from 1.97 ± 0.21 on 0d to 2.43 ± 0.24 at 5d. It was gradually declined to 1.96 ± 0.31 and 1.48 ± 0.25 at 10d and 15d respectively, representing a 25% reduction from the initial value. This early induction followed by downregulation suggests an immediate but transitory transcriptional response in the roots during Cr uptake (Fig. 4.15A).

ABCC2

Expression of ABCC2 increased consistently from 1.55 ± 0.25 at 0d to 3.31 ± 0.25 by 15d, marking a 2.1-fold upregulation. This indicates a sustained activation of ABCC2 in leaves under prolonged Cr stress that maybe associated with active vacuolar sequestration of metal ions (Fig. 4.15B).

The expression of ABCC2 in stem tissues showed a moderate upregulation from 1.18 ± 0.18 on 0d to 2.03 ± 0.43 at 10d (1.7-fold increase), followed by a decline to

1.41 ± 0.39 at 15d. This pattern suggests that intermediate role of ABCC2 in detoxification during the mid-phase of Cr exposure (Fig. 4.15B).

ABCC2 expression marked a consistent increase in root tissues, reaching 3.48 ± 0.25 at 15d showing a 1.8-fold upregulation compared to the initial value. This continuous increase in the transcript level in roots indicates the strong involvement of ABCC2 in Cr transport and sequestration at the primary site of metal uptake (Fig. 4.15B).

YSL1

A gradual and concentration-dependent increase in the YSL expression was observed in leaf tissues of *A. tenella*. The transcript level increased from 1.55 ± 0.35 at 0d to 3.95 ± 0.44 at 10d and peaked at 15d (5.31 ± 0.25), representing a 3.4-fold upregulation relative to control. This consistent upregulation suggests the activation of YSL-mediated metal transport or chelation in leaves under increasing Cr exposure which may facilitate metal redistribution and detoxification with aerial tissues (Fig. 4.15C).

The YSL transcripts in stem tissues followed a similar upregulation pattern. The expression values increased from 1.18 ± 0.18 at 0d to 4.41 ± 0.39 at 15d, marking a 3.7-fold increase over the control value. (Fig. 4.15C). The YSL gene expression in root tissues reached a maximum of 5.96 ± 0.51 at 10d, showing a three-fold increase over the control (1.97 ± 0.51). Later, it was slightly declined to 4.48 ± 0.25 at 15d (Fig. 4.15C). This progressive upregulation of YSL transporter across the treatment intervals in stem and root tissues indicates its possible role in chelation and long-distance transport of metal- nicotianamine or metal-phytosiderophore complexes from roots to leaves.

XTH32

The expression levels of XTH gene exhibited a slight increase from 1.44 ± 0.28 at 0d to 1.74 ± 0.11 at 5d, followed by a progressive downregulation to reach 0.21 ± 0.12 at 15d. This pattern suggests an initial activation of XTH to reinforce cell wall structure during the onset of metal stress and the later transcriptional suppression under prolonged Cr exposure (Fig. 4.15D). The strong suppression of XTH

transcripts approximately by 7-fold at the end treatment point indicates a possible inhibition of cell wall loosening activity, which may limit cell expansion and growth as part of stress adaptation response in plants.

Similar trend was noted in XTH expression in stem and root tissues also. In stems, a ~1.5-fold downregulation in transcript level over the initial value was observed on 15d, after a moderate increase till 10d (Fig. 4.15D). The XTH expression increased from 1.97 ± 0.05 at 0d to 2.96 ± 0.13 at 10d, marking a 1.5-fold upregulation. Later, it was sharply declined to 0.95 ± 0.002 on 15d (Fig. 4.15D). This biphasic trend suggests the temporary enhancement of XTH activity during moderate stress to maintain limited cell wall remodelling, but prolonged or high Cr exposure may suppress its function.

GST

GST expression increased progressively from 1.55 ± 0.15 at 0d to 3.95 ± 0.14 at 10d and peaked at 15d (6.31 ± 0.25) (Fig. 4.16A). This represents a four-fold increase over the control during the treatment period. A similar trend was observed in GST expression in stem tissues, though slightly lower than in the leaves. Expression increased consistently from 1.18 ± 0.18 at 0d to 2.39 ± 0.43 at 5d, 3.03 ± 0.43 at 10d, and further to 4.41 ± 0.39 at 15d (Fig. 4.16A), marking nearly 3.7-fold increase compared to the control. This time-dependent increase in GST expression with increasing Cr concentration suggests the vital role of GST in detoxification of translocated Cr through glutathione conjugation and protection of vascular tissues from oxidative damage.

GST exhibited higher induction in root tissues among all organs. Expression levels increased from 1.97 ± 0.21 at 0d to 5.96 ± 0.41 at 10d before slightly declining at 15d (4.48 ± 0.25) (Fig. 4.16A). This pronounced activation of GST in roots indicates its direct involvement in chelation and detoxification of Cr ions at the primary site of metal uptake.

PCS

PCS expression showed a gradual upregulation with increasing period of Cr exposure in *A. tenella* leaf and stem tissues. The transcript level in leaf rose from 1.55 ± 0.15 at 0d to 3.31 ± 0.25 at 15d, reflecting a 2.1-fold increase relative to control,

whereas the expressed increased from 1.18 ± 0.38 on 0d to 2.41 ± 0.49 at 15d in stem (Fig. 4.16B). This corresponds to nearly a two-fold upregulation compared to the control in both tissues. The progressive increase in PCS indicates its role in metal homeostasis and phytochelatin synthesis for Cr chelation and sequestration.

Root tissues exhibited the highest induction of PCS gene under Cr exposure. The relative expression reached 6.48 ± 0.25 at 15d, representing a 3.3-fold increase over the control which indicates strong activation of PCS synthesis under high Cr stress (Fig. 4.16B). This highlights the key role of PCS gene in PC-mediated metal detoxification, where PC-Cr complexes are formed and compartmentalized into vacuoles.

LAC6

LAC6 expression in *A. tenella* leaf showed a decreasing trend with increasing duration of Cr exposure. The relative expression slightly increased from 1.44 ± 0.28 at 0d to 1.56 ± 0.11 at 5d, but later dropped to 0.43 ± 0.12 on 15d (Fig. 4.16C). This represents the downregulation of LAC6 by ~3.3-fold over the control. The LAC6 expression followed a similar declining trend in stem tissues. After a slight increase at 5d, the transcript levels drastically declined by nearly four-fold reduction at 15d in LAC6 expression relative to control. The suppression of LAC6 expression at higher duration of Cr exposure suggests the inhibition of lignin polymerization or cell wall thickening processes in the leaf tissues, possibly due to oxidative damage or impaired metabolic activity under prolonged Cr stress.

However, LAC6 expression in root tissues exhibited a significant upregulation throughout Cr exposure. The transcript levels increased from 1.97 ± 0.21 at 0d to 2.62 ± 0.14 at 5d, and further peaked at 4.48 ± 0.25 at 15d (Fig. 4.16C). This corresponds to more than a two-fold induction over the control. This pronounced upregulation of LAC6 suggests an adaptive response involving enhanced lignin formation to reduce Cr mobility within root tissues.

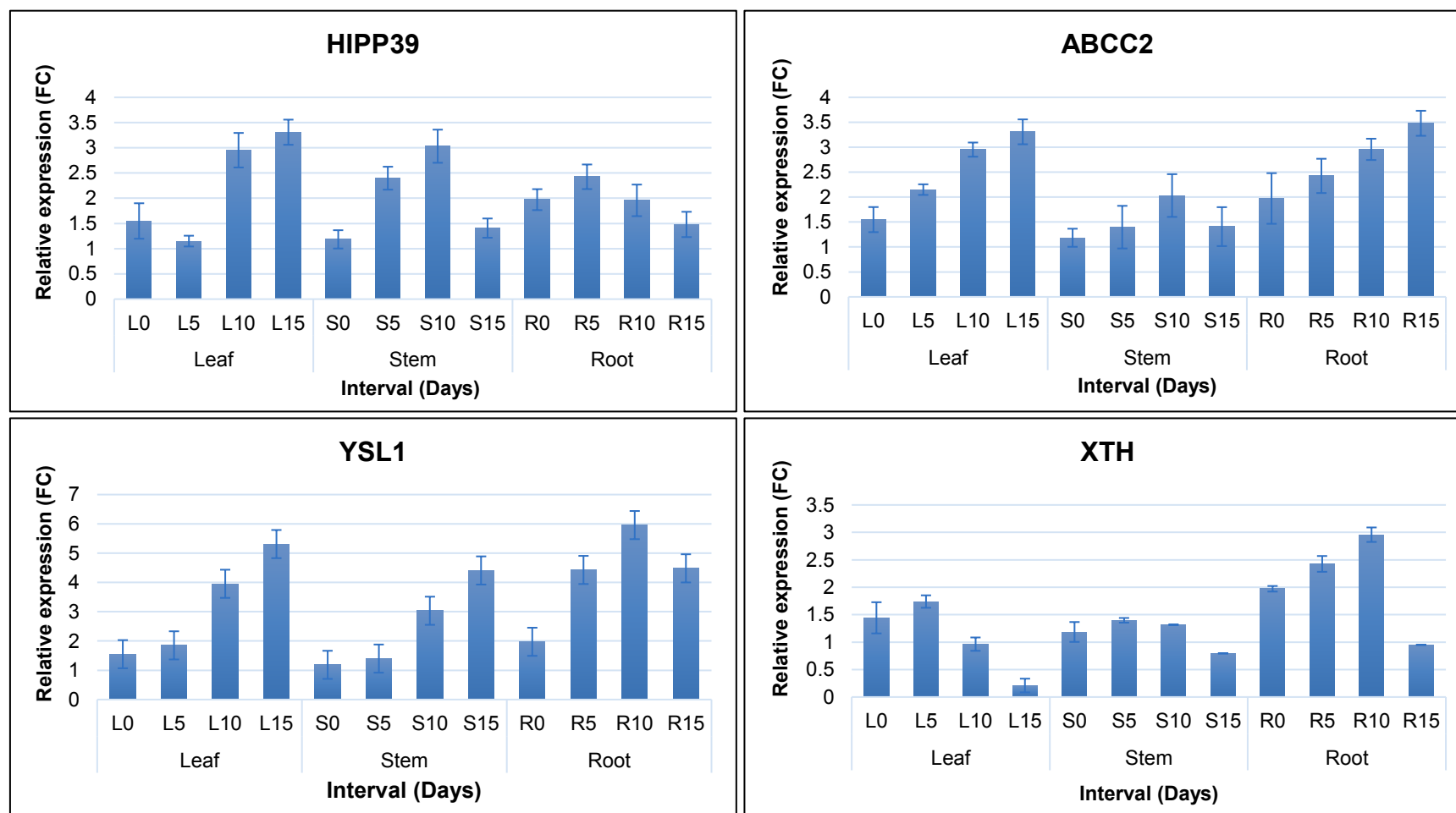


Fig 4. 15. Relative expression of HIPP39, ABCC2, YSL1 and XTH32 genes in *A. tenella* exposed to Cr treatment determined by qRT-PCR. Gene expression levels were normalized to Actin and calculated using the $2^{-\Delta\Delta C_t}$ method. Data represent the mean $\pm$ SD of three biological replicates.

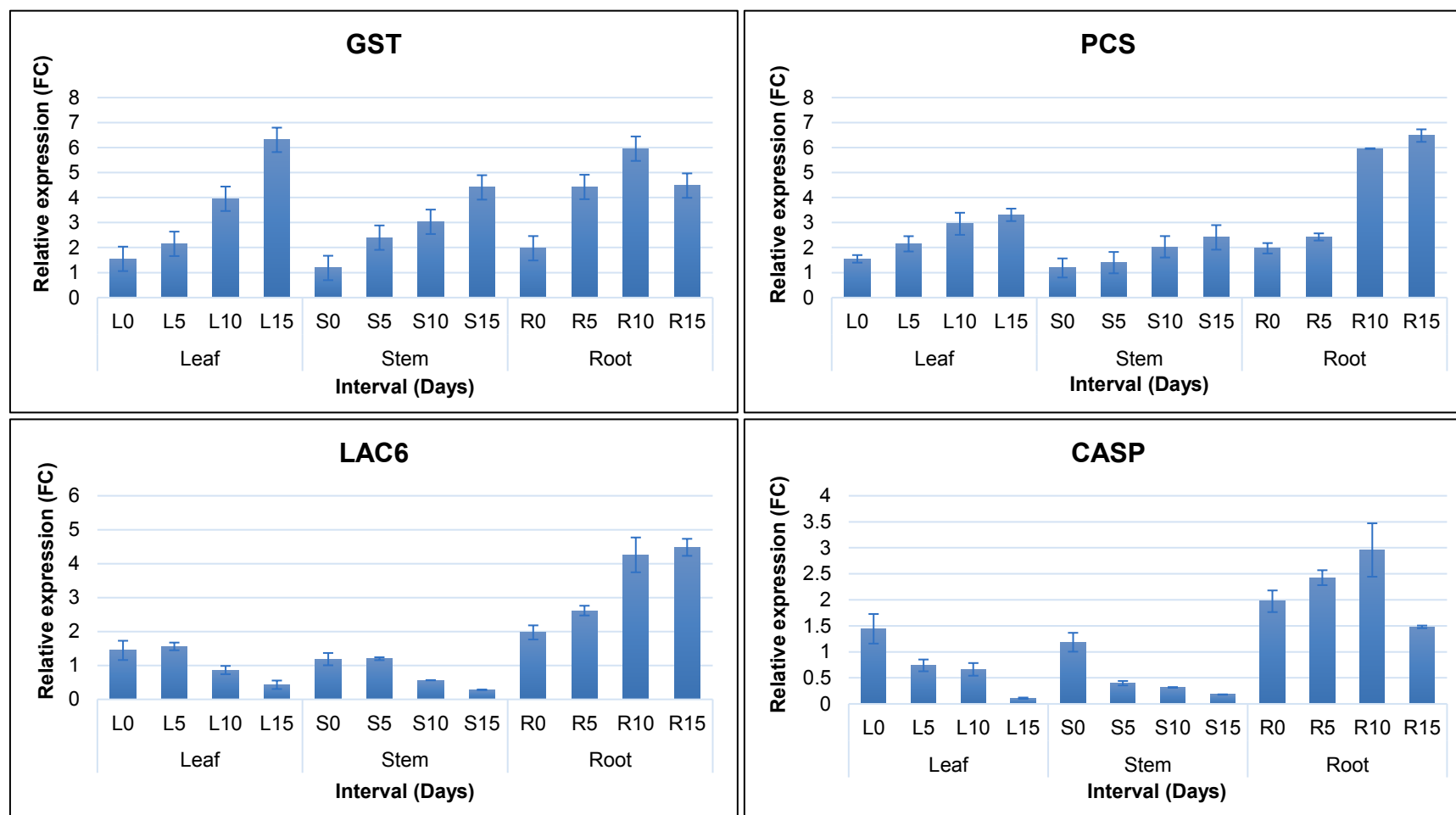


Fig 4. 16. Relative expression of GST, PCS, LAC6 and CASP genes in *A. tenella* exposed to Cr treatment determined by qRT-PCR. Gene expression levels were normalized to Actin and calculated using the $2^{-\Delta\Delta C_t}$ method. Data represent the mean $\pm$ SD of three biological replicates.

CASP

CASP expression in leaf and stem tissues exhibited a significant downregulation with increasing stress duration. Expression levels of CASP in leaf and stem decreased respectively from 1.44 ± 0.28 and 1.18 ± 0.18 at 0d to reach the lowest level of 0.11 ± 0.01 and 0.18 ± 0.004 by 15d (Fig. 4.16D). This corresponds to downregulation of CASP activity in leaf by ~13-fold and in stem by ~6-fold. This consistent decline indicates the reduction of CASP-associated membrane reinforcement in these tissues.

In root tissues, CASP expression was upregulated in the early and mid-stages of Cr exposure. The relative expression increased from 1.97 ± 0.21 at 0d to 2.43 ± 0.14 at 5d and reached 2.96 ± 0.51 at 10d (Fig. 4.16D). It later declined to 1.48 ± 0.03 at 15d, which suggests that CASP activation in roots is primarily an early defensive response that stabilizes during prolonged Cr exposure.

4.5. Discussion

4.5.1. Metabolomic analysis of *A. tenella* leaves under Cr stress

The metabolomic reprogramming of *A. tenella* under chromium stress reflects a complex defense system that enables the plant to tolerate metal toxicity by coordinating physiological, biochemical, and molecular adaptations. This strategy involves the cell wall modification, metal transport, chelation and sequestration, antioxidant defense, hormonal regulation, and shifts in photosynthetic system.

Cell wall metabolism was primarily altered under Cr exposure in *A. tenella*, as in case of any heavy metal stress. Phenylpropanoid pathway metabolites like caffeic acid conjugates and flavonoids that contribute to cell wall remodeling showed differential regulation. These metabolites provide functional groups (hydroxyl, carboxyl) that bind and immobilize Cr ions in the cell wall, restricting their movement and apoplastic entry and thereby preventing their excess uptake into the cells (Aluko *et al.*, 2025). Feruloyl esters can cross-link with polysaccharides strengthening the structural integrity and immobilizing metals at the apoplast.

However, Cr stress induced strong downregulation of caffeic acid 3-O-glucuronide and N-feruloyltyramine in *A. tenella*, which indicates a reduced lignin or phenylpropanoid incorporation into the cell wall (del Rio *et al.*, 2020; Kisa *et al.*, 2016). The stilbene precursors (1,2-dihydrostilbene) and some saponins under Cr stress were also downregulated indicating a metabolic shift prioritizing cytosolic detoxification (metal chelation) and oxidative stress defense (Aluko *et al.*, 2025). This differential response of phenolic metabolites contradicts with the common stress response strategies in plants, where the cell wall strengthening acts as the primary defense against metal entry. However, the upregulation of O-feruloyl galactarate and 6-pentadecyl salicylic acid reflects an attempt in reinforcing the cell wall and enhancing stress resistance. Salicylic acid derivatives can modulate lignin deposition and cell wall fortification, limiting heavy metal permeation (Jia *et al.*, 2021).

Chromium induces oxidative stress by increasing reactive oxygen species (ROS) which damage proteins, lipids, and DNA. *A. tenella* counters this stress by significant enhancement of antioxidant metabolites, especially flavonoids such as quercetin, rutin, kaempferol, and isorhamnetin derivatives. Their ability to scavenge ROS directly and chelate metal ions aids them play a key role in metal detoxification (Matsia *et al.*, 2022). The upregulated flavonoids (apigenin, auriculoside, calendoflaside) not only scavenge ROS but also directly chelate Cr through hydroxyl and carbonyl groups. The significant upregulation of quercetin glycosides (quercetin-3-glucoside and rutin) highlights flavonoids as the key metabolites involved in detoxification. They are highly water-soluble, can chelate Cr ions through hydroxyl and keto groups and efficiently transport Cr-flavonoid complexes to vacuoles for sequestration. These metabolites can also scavenge ROS to reduce oxidative damage caused by Cr-induced redox cycling (Abdullah *et al.*, 2024; Matsia *et al.*, 2022).

Caffeic acid conjugates and other antioxidants like carotenoid derivatives (e.g., apo-10'-violaxanthal) and chlorophyll degradation products (pheophytin a) further contribute to ROS scavenging and membrane stabilization (Saud *et al.*, 2022).

Caffeic acid conjugates are potent metal binders. Caffeic acid 3-O-glucuronide was significantly decreased which suggests the selective channeling toward specific antioxidant conjugates better suited for Cr detoxification. In addition to the photosystem stress, significant accumulation of pheophytin and related tetrapyrroles indicates the additional antioxidant and metal-binding roles. Geranyl acetoacetate (a terpenoid precursor) may play a role in generating terpenoid antioxidants that protects membranes. These observations confirm that *A. tenella* prioritizes antioxidant flavonoids and phenylpropanoids as primary defenses against Cr-induced oxidative bursts and thereby preserve cellular function.

A key mechanism of heavy metal tolerance in plants is the vacuolar compartmentalization of toxic ions to prevent the metal interference with cellular processes and to limit oxidative damage inside the cytoplasm. The metabolomic changes in *A. tenella* pointed to the active transport of Cr ions and vacuolar sequestration strategies. Organic acid derivatives such as 6-Epi-7-isocucurbitic acid glucoside were strongly induced, consistent with their role in metal chelation and facilitating vacuolar storage of Cr complexes (Huda *et al.*, 2021). The strong upregulation of flavonoid glycosides like rutinoides and hexosides indicates glycosylation as a mechanism to increase water solubility and facilitate vacuolar storage (Matsia *et al.*, 2022). The moderate induction of alkaloids such as retronecine (pyrrolizidine alkaloid) and sophoranone implies a supplementary role as chelators in vacuolar detoxification. However, downregulation of nitrogen-rich alkaloids (e.g., carnegine, matrine) suggests a redirection of nitrogen metabolism away from secondary alkaloid biosynthesis toward glutathione and phytochelatin synthesis, both essential for vacuolar metal sequestration. These metabolic shifts together suggest that chelation and vacuolar compartmentalization by flavonoids, organic acids and glutathione-derived metabolites is the central Cr sequestration system in *A. tenella*.

Phytohormones tightly regulate stress responses in plants. Metabolites linked to hormonal pathways revealed significant reprogramming of stress signalling under Cr stress. Downregulation of jasmonate derivatives (7-Epi-12-hydroxyjasmonic acid

glucoside, Beta-D-glucopyranosyl-11-hydroxyjasmonic acid) suggests the suppression of JA-mediated pathways for the potential conservation of resources by redirecting metabolic flux toward phenylpropanoids and flavonoids, prioritizing chelation and ROS detoxification over growth-related stress responses (Barzin *et al.*, 2022). Instead, salicylic acid derivatives were upregulated consistent with their known roles in activating antioxidant enzymes and enhancing heavy metal tolerance (Gupta *et al.*, 2021; Yang *et al.*, 2021). The sharp decline of auxin-related indolic metabolites including tryptophan and indole acrylic acid reflects a shift away from growth-related signaling toward stress prioritization. Interestingly, flavonoids such as rutin and quercetin also function as signaling molecules that can modulate expression of genes involved in antioxidant enzyme production and stress adaptation. Reduced levels of saponins and alkaloids indicate a metabolic shift to abiotic detoxification over biotic stress defense. These results highlight the hormonal rebalancing that efficiently shifts the metabolism of plant from growth to detoxification and survival.

Exposure to Cr caused profound changes in membrane lipid composition which reflects both structural changes and activation of lipid-based signaling. Phosphatidylinositol (PI) derivatives including PI (20:3/18:1) were strongly upregulated. This triggers the activation of phosphoinositide signaling cascades that regulate vesicle trafficking and transporter activity essential for directing metal-chelate complexes into vacuoles (Choudhary *et al.*, 2024). However, major structural lipids like phosphatidylcholine (PC) were strongly downregulated, indicating turnover and redistribution of lipid pools toward PI-based signaling lipids. Such membrane remodeling suggests enhanced membrane integrity under oxidative stress and efficient facilitation of intracellular metal detoxification processes.

Pigment-related metabolites showed significant changes. The strong induction of Pheophytin a and pyropheophorbide derivatives indicates the chlorophyll degradation. This shift highlights the impact of Cr on the stability of photosynthetic apparatus. Along with the oxidative damage, the accelerated chlorophyll

breakdown may also represent a protective strategy as it induces photo-oxidative stress by limiting light capture under stress conditions. The accumulation of breakdown products further implies an active detoxification mechanism, where damaged pigments are rapidly degraded to prevent the accumulation of phototoxic intermediates (Tan *et al.*, 2025; Harpaz-Saad *et al.*, 2007).

A. tenella, thus, exhibits a well-coordinated metabolic adjustment to Cr stress integrating structural, chemical, and signaling mechanisms consistent with known heavy metal tolerance processes in plants. This involved the cell wall reinforcement, antioxidant activation, modulation of growth and defense signaling, active cellular compartmentalization, and membrane lipid remodeling. This integrated strategy suggests the metabolic plasticity of *A. tenella* that enables it to balance growth suppression with survival under Cr stress.

4.5.2. Transcriptome analysis of *A. tenella* leaves under Cr stress

Chromium (Cr) is a toxic heavy metal that significantly impairs plant growth, metabolism, and cellular homeostasis. No specific transporters have been yet identified for the uptake and transport of Cr in plants. Nevertheless, many transporters were involved in the transfer processes of Cr uptake, transport, chelation and sequestration in *A. tenella*. These transporters include heavy metal transporters, ATP-binding cassette (ABC) transporters, ZRT, IRT-like proteins (ZIPs), Natural Resistance-Associated Macrophage Proteins (NRAMPs), Cation Diffusion Facilitators (CDFs), MATE efflux transporters, Metal Tolerance Proteins (MTPs), Plant Cadmium Resistance (PCR) proteins, vacuolar iron transporters (VITs), and Heavy Metal-Associated Isoprenylated Plant Proteins (HIPPs). Additionally, sulfate and YSL transporters were implicated in Cr transport and detoxification pathways.

The ZIP family of transporters, which mediate the uptake of Zn, Fe, Mn, and other divalent metal ions, were significantly upregulated under Cr stress in *A. tenella*. ZIP1, ZIP2, ZIP4, ZIP6, ZIP7, and ZIP29 were found to be overexpressed, suggesting their roles in Cr transport. ZIP proteins are primarily responsible for

moving Zn and Fe ions into the cytoplasm, but their promiscuous nature allows the transport of other metals like Cd and Ni. In *Morus alba*, similar ZIP genes facilitated Cd transport (Guo *et al.*, 2021), indicating that Cr ions may similarly exploit ZIP transport pathways for cellular entry. Furthermore, IRTs, which are key in metal ion uptake, were reported to facilitate the transport of Zn, Mn, and Cd in *A. thaliana* (Ajeesh *et al.*, 2020). The upregulation of ZIP and IRT genes in *A. tenella* highlights their possible role as conduits for Cr influx into root and leaf cells. Vacuolar sequestration is a critical detoxification mechanism for excess heavy metals. Vacuolar Iron Transporter (VIT) proteins, VIT1, VIT2, and VIT4 were significantly upregulated in *A. tenella* under Cr stress, suggesting that these transporters aid in Cr sequestration into vacuoles to mitigate cytosolic toxicity. AtVIT1 and OsVIT2, are known to transport Fe and Mn into vacuoles (Che *et al.*, 2021; Zhu *et al.*, 2016). The role of VITs in Cr detoxification may involve similar mechanisms where Cr is compartmentalized into vacuoles, thereby reducing its bioavailability in the cytoplasm.

ABC transporters play a prominent role in metal detoxification by exporting toxic ions and facilitating vacuolar sequestration. In *A. tenella*, genes such as ABCG36, ABCB11, ABCC2, ABCG11, ABCC3, and ABCG5 were significantly upregulated under Cr stress. ABCC transporters are particularly crucial as they mobilize metal-phytochelatin (PC) complexes into vacuoles. For example, AtABCC1 and AtABCC2 facilitate the sequestration of Cd and As into vacuoles in *Arabidopsis* (Mendoza-Cózatl *et al.*, 2010). The upregulation of ABCC transporters in *A. tenella* suggests a similar role in Cr-PC complex sequestration, contributing to Cr detoxification. ABCG and ABCB transporters are involved in heavy metal efflux and glutathione-dependent detoxification pathways. AtPDR12 (ABCG40) in *Arabidopsis* is associated with Pb resistance (Lee *et al.*, 2005), while ABCG36 homologs enhance Cd resistance in rice and *Arabidopsis* (Reemmer, 2014). The upregulation of ABCG and ABCB genes in *A. tenella* indicates their involvement in Cr efflux and cellular detoxification.

The Multidrug and Toxic Compound Extrusion (MATE) transporters are critical for detoxifying harmful compounds, including heavy metals. In *A. tenella*, 168 MATE genes were differentially expressed, with upregulation observed in MATE48, MATE12, MATE35, MATE45, and MATE27. MATE proteins, particularly Group 2 MATEs, are known for their roles in Al detoxification through citrate efflux (Du *et al.*, 2021). For instance, *Arabidopsis* MATE and barley HvAACT1 facilitate Al resistance by transporting organic acids (Chen *et al.*, 2020). The differential expression of MATE genes in *A. tenella* suggests a role in Cr detoxification, likely by exporting Cr-organic acid complexes or sequestering Cr into cellular compartments.

PCR proteins facilitate metal efflux, while MTPs mediate divalent cation transport to maintain metal homeostasis. In *A. tenella*, PCR2 and PCR10 were significantly downregulated under Cr stress, which might indicate reduced efflux activity and an increased Cr influx into leaf cells for detoxification. Similar patterns were observed in *Populus* and *Sedum* species under Cd and Al stress (Lin *et al.*, 2020; Guan *et al.*, 2023). The downregulation of PCR genes may be part of a strategic shift to store Cr in specific compartments for detoxification. MTP genes in *A. tenella* likely play a role in Cr sequestration and transport to vacuoles or leaves, mirroring their functions in other plants. AtMTP1 and OsMTP1 translocate Cd, Zn, and Co to maintain metal homeostasis (Desbrosses-Fonrouge *et al.*, 2005; Menguer *et al.*, 2013). Upregulated MTPs in *A. tenella* could similarly regulate Cr compartmentalization, ensuring cellular metal homeostasis under stress.

NRAMP family transporters, including Nramp3, Nramp5, and Nramp6, were upregulated in *A. tenella* under Cr stress, suggesting their roles in Cr uptake, translocation, and detoxification. NRAMPs are known for their broad substrate specificity, transporting Fe, Mn, and Cd in rice, potato, and *Sedum* species (Sasaki *et al.*, 2012; Tian *et al.*, 2021). For example, AtNramp3 in *Arabidopsis* mediates vacuolar Fe and Mn balance under metal stress (Lanquar *et al.*, 2010). The upregulation of NRAMPs in *A. tenella* indicates their importance in Cr transport and vacuolar sequestration, helping to maintain cellular metal homeostasis. YSL transporters,

which mediate the transport of metal-nicotianamine (NA) complexes, were also implicated in Cr detoxification. High expression of nicotianamine synthase (NAS) in *A. tenella* under Cr stress suggests enhanced formation of NA-Cr complexes, which are likely transported via YSL proteins. Similar roles of YSL transporters have been reported in enhancing Cd tolerance in *M. sinensis* and *A. thaliana* (Seregin and Kozhevnikova, 2023). HIPPs act as metal-binding chaperones that facilitate metal sequestration and immobilization. HIP39 and HIP24 were significantly upregulated in *A. tenella*, suggesting their involvement in Cr binding and detoxification. HIPs like HIP26 bind Cd in *Arabidopsis* (Gao *et al.*, 2009), while ZmHIP aids Pb deposition in cell walls (Ma *et al.*, 2022). The upregulation of HIPs in *A. tenella* may reflect their role in Cr sequestration in cell walls, reducing cytosolic Cr toxicity. Sulfate transporters are known to mediate Cr(VI) uptake in plants. OsSultr1;2 in rice facilitates Cr uptake, and its knockout reduces Cr accumulation (Xu *et al.*, 2021). In *A. tenella*, sulfate transporters likely mediate Cr(VI) uptake, underscoring their role in Cr stress responses.

The cell wall and its components (cellulose, hemicellulose, pectin, and lignin) act as the first line of defense against Cr toxicity by preventing Cr entry into the cytoplasm. The negatively charged functional groups (-COOH, -OH, and -SH) in these components effectively bind Cr ions, thereby reducing their mobility and toxicity (Antoniadis *et al.*, 2017; Luo *et al.*, 2016). This mechanism has also been observed in *Morus alba* and other high Cd-accumulating plants, where enhanced cell wall biosynthesis and modification increase metal tolerance (Zhou *et al.*, 2016; Guo *et al.*, 2021). Cellulose and hemicellulose, as essential structural components of the primary and secondary cell walls, plays a critical role in Cr detoxification through its ability to bind metal ions via hydroxyl (-OH) groups. The upregulation of genes involved in cellulose biosynthesis, such as cellulose synthase (CS) under Cr stress suggests active cellulose biosynthesis to strengthen the cell wall, thereby limiting Cr mobility and penetration into the cytoplasm. This aligns with the findings of Liu *et al.* (2017), who reported that upregulation of CS genes facilitated Cd binding through cellulose synthesis in *Cosmos bipinnatus*. The increased cellulose synthesis in *A. tenella* likely compensates for other weakened barriers,

contributing to Cr sequestration and immobilization within the cell wall. Xyloglucan endotransglucosylase/hydrolase (XTH) enzymes play a key role in cell wall remodelling by modifying xyloglucan strands and facilitating their integration into cellulose microfibrils. In *C. bipinnatus*, upregulation of XTH genes under Cd stress enhanced cell wall rigidity and metal immobilization (Liu *et al.*, 2017). However, in *A. tenella*, significant downregulation of XTH genes was observed in response to Cr stress. This reduction in XTH expression suggests a diminished capacity for active xyloglucan remodelling, potentially impacting the dynamic restructuring of the cell wall. Interestingly, plasmodesmata callose-binding protein (PDCB) genes were also significantly downregulated under Cr stress which indicates a reduced ability for callose deposition. Callose, a β -1,3-glucan, functions as a mechanical barrier by blocking symplastic movement of metal ions through plasmodesmata, preventing their entry into the protoplasts. Its downregulation suggests decreased formation of such barriers, potentially allowing increased Cr mobility within the plant tissues.

Pectin, a key component of the primary cell wall, plays a critical role in heavy metal sequestration due to its carboxyl (-COOH) groups, which chelate metal ions such as Cr. The activity of pectin methylesterase (PME) is crucial in this process, as it reduces the methylation of pectin that increases the availability of ionic soluble pectin (ISP) for Cr binding (Muschitz *et al.*, 2015; Wu *et al.*, 2020). However, the downregulation of pectin methylesterase inhibitors (PMEIs) in our study presents an interesting dynamic. PMEIs typically regulate PME activity by inhibiting excessive demethylation of pectin, thereby maintaining a balance between highly methylated and demethylated pectin. The observed downregulation of PMEIs could result in unregulated PME activity leading to increased demethylation of pectin. This outcome could be viewed as an adaptive response to Cr stress, as it increases the number of free carboxyl groups available for Cr chelation, thereby enhancing the cell wall's ability to immobilize Cr ions and reduce their toxicity. However, pectin acetylerase (PAE) and PME genes were downregulated, which shows that pectin stays more methylated or acetylated which is less able to form metal-pectate crosslinks. This tends to limit metal immobilization, and hence metals

can move freely toward the cell. Contrasting mechanism was reported in *Morus alba* under Cd stress, where upregulation of pectin biosynthesis and PME activity contributed to Cd accumulation in the cell wall (Guo *et al.*, 2021).

Lignin is a crucial component of the cell wall that enhances its rigidity and serves as a physical barrier to limit heavy metal mobility. It also sequesters metals like Cr through functional groups such as hydroxyl (-OH), carboxyl (-COOH), and methoxy (-OCH₃), reducing their translocation into the symplast (Parrotta *et al.*, 2015). The phenylpropanoid biosynthesis pathway, which governs lignin synthesis, is often enriched under heavy metal stress. In species like *Typha orientalis* under Pb stress, upregulation of lignin biosynthesis genes resulted in increased cell wall thickness and metal sequestration (Xu *et al.*, 2019). However, in *A. tenella* under Cr stress, the observed downregulation or non-significant upregulation of lignin biosynthesis genes (such as 4CL, HCT, CCoAOMT, LAC, and POD) suggests a unique or altered response compared to other plant species. Under Cr stress, *A. tenella* may prioritize other detoxification strategies, such as pectin modification, cellulose remodeling, or vacuolar sequestration, rather than allocating resources to lignin synthesis. Cr(VI) toxicity can inhibit enzymes involved in lignin biosynthesis and disrupt the phenylpropanoid pathway. Unlike Pb or Cd, which are commonly immobilized by lignin deposition in the cell wall, Cr may be preferentially sequestered through pectin de-methylation and cellulose remodeling in *A. tenella*. The downregulation or insignificant upregulation of lignin-related genes may result from oxidative stress, post-transcriptional regulation, or feedback inhibition within the phenylpropanoid pathway. Cr stress might induce negative regulation to limit lignin biosynthesis and redirect metabolic flux toward other pathways crucial for stress adaptation. This species-specific strategy highlights the plasticity of cell wall modification mechanisms in plants under varying heavy metal stresses.

The downregulation of sucrose synthase (SUS), cellulose synthase (CS), PME and PAE genes suggests decreased pectin remodeling and availability of de-acetylated pectin which lowers Cr binding capacity of the cell wall. In addition, the downregulation of Casparian strip membrane proteins (CASPs), polygalacturonase

(PG), laccase, and expansin genes indicates a reduced focus on lignin deposition, Casparian strip reinforcement, and cell wall loosening. This may again reflect a prioritization of energy and resources toward other pathways which are more critical for Cr tolerance in *A. tenella*. The downregulation of PG also suggests limited degradation of pectin and thereby maintaining structural integrity of the cell wall that favors metal immobilization. The downregulation of DEGs encoding PAE, GME, and UGT may indicate a finely balanced regulation of these enzymes to maintain pectin and hemicellulose structure without excessive remodeling. GME and UGT, involved in glycan biosynthesis and sugar metabolism, may play secondary roles under Cr stress, with other mechanisms compensating for their limited activity. Overall, the results indicated the decreased formation of mechanical barriers in cell walls of *A. tenella* such as pectin and callose deposition and lignification, potentially allowing increased Cr mobility into the protoplasts under Cr stress.

After Cr entering cytoplasm, it would be bound with metal chelates and then sequestered into vacuoles to reduce their toxicity. Glutathione (GSH), a sulfur-rich tripeptide composed of glutamate, cysteine, and glycine, plays a key role in Cr detoxification due to its ability to chelate metal ions through its thiol group, forming stable Cr-GSH complexes. In *A. tenella*, 430 differentially expressed genes (DEGs) were annotated to GSH-related pathways, including those involved in GSH synthesis, utilization, and recycling. Genes encoding gamma-glutamyl cysteine synthetase (GCS), the rate-limiting enzyme in GSH biosynthesis, and gamma-glutamyl transpeptidase (GGT), which facilitates GSH breakdown and recycling, were upregulated. This suggests an increased GSH production under Cr stress to meet the demands for chelation and oxidative stress mitigation (Xu *et al.*, 2018). The activity of GGT is particularly critical as it ensures the availability of GSH precursors for phytochelatin (PC) synthesis, which chelates Cr into less toxic forms (Zhou *et al.*, 2016). Interestingly, glutathione synthetase (GSS), responsible for the final step of GSH synthesis, was also upregulated. This reflects an enhanced capacity for GSH production to support Cr chelation and antioxidant defense mechanisms. Similar findings were reported in *Triticum aestivum*, where sulfur

assimilation and GSH biosynthesis were enhanced under cadmium (Cd) stress (Khan *et al.*, 2007). In contrast, glutathione hydrolase (GH) was downregulated, possibly to limit GSH breakdown and conserve resources during stress conditions. The glutathione S-transferases (GSTs), a family of detoxifying enzymes, were significantly upregulated in *A. tenella* under Cr stress. GSTs catalyse the conjugation of GSH to Cr ions, forming GSH-Cr complexes that are less toxic and better suited for sequestration into vacuoles (Huang *et al.*, 2019; Yan *et al.*, 2019). In addition to their detoxification role, GSTs participate in oxidative stress mitigation by scavenging reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂). The upregulation of glutathione peroxidase 8 (GPX8) further supports this antioxidant defence mechanism. GPX8 detoxifies lipid peroxides and H₂O₂ using GSH as a substrate, thereby maintaining cellular redox homeostasis (Yan *et al.*, 2019). This is consistent with findings in *Brassica napus*, where GST and GPX upregulation enhanced tolerance to Cd stress (Ding *et al.*, 2018). Sulphur assimilation is critical for supporting GSH production under Cr stress.

In *A. tenella*, genes associated with sulphur metabolism, including serine acetyltransferase (SAT1, SAT5) and ATP sulfurylase (ATPS), were upregulated. These enzymes play key roles in the assimilation of sulphur and the synthesis of cysteine, a precursor for GSH (Xu *et al.*, 2018). The enhanced expression of sulphur assimilation genes mirrors similar responses in *Sorghum bicolor* and *Triticum aestivum* under heavy metal stress, where increased ATPS activity promoted sulfur metabolism and improved Cd tolerance (Akbudak and Filiz, 2019; Khan *et al.*, 2007). The upregulation of cysteine synthase further supports the increased biosynthesis of sulphur-containing amino acids. Additionally, the upregulation of ornithine decarboxylase, an enzyme involved in polyamine synthesis, suggests a coordinated response to sustain GSH synthesis while maintaining cellular energy balance under Cr stress. GSH also serves as a precursor for phytochelatins (PCs) and metallothioneins (MTs), two critical sulphur-rich molecules involved in Cr chelation and sequestration. The upregulation of glutathione gamma-glutamylcysteinyltransferase 1 and glutathione gamma-glutamylcysteinyltransferase 3 strongly indicates enhanced PC synthesis. PCs chelate Cr

ions into PC-Cr complexes, reducing their bioavailability and toxicity. This mechanism highlights the role of phytochelatins as a critical pathway for Cr detoxification, in line with observations in other plants like *Brassica napus* and *Morus alba* under Cd stress (Guo *et al.*, 2021; Zhou *et al.*, 2016). NAD(P)H-dependent dehydrogenase FQR1 was upregulated in *A. tenella*, indicating the reduction of Cr(VI) to Cr(III) reduction in the cytoplasm.

Metallothioneins (MTs), small cysteine-rich proteins, were upregulated, particularly MT2 genes. MTs bind Cr ions through their thiol groups, forming stable Cr-MT complexes. This reduces Cr toxicity and facilitates its sequestration into vacuoles. Similar responses have been observed in *M. sinensis* and *Chara australis* under heavy metal stress (Nie *et al.*, 2021; Clabeaux *et al.*, 2011). Vacuolar sequestration of GSH-Cr and PC-Cr complexes is a critical detoxification mechanism. In *A. tenella*, genes encoding ATP-binding cassette (ABC) transporters, particularly the ABCC subfamily (ABCC1, ABCC2, ABCC3), were significantly upregulated under Cr stress. ABCC transporters mediate the active transport of Cr-GSH and PC-Cr complexes into vacuoles, effectively reducing cytoplasmic Cr toxicity (Guo *et al.*, 2021; Yan *et al.*, 2019). The vacuolar sequestration mechanism aligns with findings in *P. americana*, *A. hypogaea* and *C. bipinnatus*, where heavy metal detoxification predominantly relies on vacuolar compartmentalization (Liu *et al.*, 2017). Several DEGs were annotated as iron-sulphur protein-related genes, which are essential for cellular processes such as electron transport. Under Cr stress, these proteins are vulnerable to oxidative damage. Their differential expression suggests an adaptive response to maintain iron homeostasis and protect iron-sulphur clusters from oxidative stress. This further underscores the role of GSH in safeguarding cellular components during Cr toxicity.

Chromium stress induces the overproduction of reactive oxygen species (ROS), which can cause oxidative damage to cellular components, including lipids, proteins, and nucleic acids (Jiang *et al.*, 2020; Xu *et al.*, 2019). Enhanced activities of antioxidant enzymes, including SOD, CAT, POD, APX, and GST, were observed in response to Cr stress, demonstrating their critical role in ROS scavenging and

oxidative damage mitigation. SOD is the first line of defense against oxidative stress, converting superoxide radicals (O_2^-) into H_2O_2 and oxygen. The upregulation of SOD genes observed in present study suggests an active scavenging of O_2^- under Cr stress. However, H_2O_2 itself is a harmful byproduct that must be further detoxified. Catalase (CAT) and peroxidases (POD) play a pivotal role in this process by converting H_2O_2 to water and oxygen. CATs have high turnover rates for H_2O_2 , whereas PODs exhibit a higher affinity for H_2O_2 and are often more responsive to stress conditions (Xu *et al.*, 2019; Gupta *et al.*, 2013). The substantial upregulation of POD-encoding genes in *A. tenella* indicates the enzyme's crucial role in mitigating oxidative damage under Cr stress. Similarly, the increased activity of APX, which catalyzes the conversion of H_2O_2 to water using ascorbate as a reducing agent underscores its importance in maintaining cellular redox homeostasis. Genes encoding enzymes involved in the GST and AsA-GSH cycle, including glutathione peroxidase (GPX), glutathione reductase (GR), and dehydroascorbate reductase (DHAR). This cycle is essential for the elimination of ROS and the regeneration of reduced ascorbate and glutathione (GSH), two critical non-enzymatic antioxidants (Liu *et al.*, 2017; Xu *et al.*, 2019). Enhanced activity of GSH peroxidase and GR observed in this study indicates an active AsA-GSH cycle, contributing to improved Cr tolerance in *A. tenella*. Similar observations have been reported in *C. bipinnatus* under Cd stress, where the upregulation of AsA-GSH cycle genes improved Cd tolerance (Liu *et al.*, 2017).

In addition to antioxidant enzymes, other detoxification-related enzymes, such as cytochrome P450 (CYPs) and glutathione S-transferases (GST), were significantly upregulated in *A. tenella* under Cr stress. Cytochrome P450s are known for their involvement in the detoxification of heavy metals (HMs) and other xenobiotics (Rai *et al.*, 2015). For instance, Liu *et al.* (2009) identified altered expression of 28 CYPs in *Arabidopsis* under lead (Pb) stress, while the overexpression of CYP450-like genes enhanced HM tolerance in plants. The upregulation of CYPs in *A. tenella* suggests their role in Cr detoxification through altered regulatory networks. Glutathione S-transferases (GSTs) also play a critical role in ROS scavenging and detoxification of Cr by conjugating GSH to toxic molecules (Xu *et al.*, 2019; Kumar and Majeti, 2014).

Upregulation of GST genes under Cr stress in this study highlights their involvement in Cr tolerance mechanisms. Heat shock proteins (HSPs) are known to protect plants from adverse environmental conditions, including heavy metal stress (Feng *et al.*, 2019). The upregulation of 75% HSPs in the present study suggests their role in protein stabilization and refolding, thus preventing cellular damage under Cr-induced oxidative stress in *A. tenella*. Similar findings have been reported in switchgrass and cherry tomato under Cd stress (Su *et al.*, 2021; Song *et al.*, 2018). Lipid peroxidation is a key indicator of oxidative stress in plants, and malondialdehyde (MDA) is its primary byproduct. In the physiological studies, Cr stress increased MDA concentrations in *A. tenella* leaves, reflecting significant oxidative damage. The observed enhancement in antioxidant enzyme activities, such as CAT, POD, SOD, and APX, demonstrates the plant's ability to mitigate Cr-induced lipid peroxidation and ROS accumulation. This coordination among different antioxidant enzymes plays a critical role in alleviating oxidative stress and enhancing Cr tolerance.

The results also highlighted the intricate interplay between phytohormone signaling pathways and kinase-mediated signaling cascades in *A. tenella* under chromium (Cr) stress. The MAPK signaling cascade emerged as a key regulator of Cr stress responses, with a significant upregulation of various kinases and transcription factors. Notably, MAPK, WRKY transcription factors (WRKY22 and WRKY33), MYC2, and ethylene-responsive factor 1 (ERF1) were upregulated, suggesting their roles in activating stress-adaptive mechanisms and promoting defense responses. Furthermore, genes such as aminocyclopropane-1-carboxylate synthase (ACS), mitogen-activated protein kinase kinases 4/5 (MKK4/5), serine/threonine-protein kinase OXI1, MKK9, and respiratory burst oxidase (RBOH) were upregulated, highlighting the MAPK cascade's involvement in cellular stress responses, signal amplification, and ROS regulation (Singh *et al.*, 2016; Luo *et al.*, 2016). Calcium signaling genes, including mitochondrial calcium uniporter (MCU), phosphatidylinositol phospholipase C delta (PLCD), calcium-dependent protein kinase (CPK), and voltage-dependent anion channel protein 2 (VDAC2), were also upregulated, with VDAC2 showing an exceptionally high fold

change. This suggests that calcium signaling works in conjunction with MAPK pathways to trigger downstream stress responses and enhance Cr tolerance (Qiao *et al.*, 2016; Huang and Huang, 2008).

Phytohormones, particularly abscisic acid (ABA), jasmonic acid (JA), and ethylene (ETH), played vital roles in modulating the MAPK pathway to enhance Cr stress tolerance in *A. tenella*. ABA biosynthesis genes were significantly upregulated under Cr stress, reflecting the central role of ABA in mediating stress adaptation mechanisms such as stomatal closure and osmotic balance. Genes like phosphatase 2C (PP2C), MAPKKK18-like, and catalase (CAT1), which are part of the ABA-MAPK signaling cascade, were upregulated, aiding the plant in combating oxidative stress and cellular damage caused by Cr exposure (Zhao *et al.*, 2014; Guo *et al.*, 2021). Heavy metal toxicity is known to activate both ABA-dependent and ABA-independent signaling pathways in plants. In *A. tenella* the observed upregulation of AREB and DREB genes, MYC/MYB transcription factors and ABA-biosynthetic genes implies that *A. tenella* relies on both ABA-dependent and ABA-independent pathways of stress response. Ethylene signaling demonstrated a more complex regulation. While ethylene biosynthesis genes such as ACS and ACC oxidase (ACO) were downregulated, signaling components like ethylene receptor (ETR), ethylene-insensitive 3 (EIN3), and ERF1 were upregulated, indicating that ethylene signaling remained active despite reduced ethylene production. This likely represents an adaptive mechanism to maintain stress responses and defense pathways under Cr stress (Keunen *et al.*, 2016; Schellingen *et al.*, 2015).

In the jasmonic acid signaling pathway, the upregulation of MYC2 and jasmonate ZIM domain-containing proteins (JAZ) indicated the activation of JA-mediated stress responses. JA plays a crucial role in promoting defense mechanisms and modulating plant growth under abiotic stress conditions, including heavy metal stress (Wu *et al.*, 2015; Luo *et al.*, 2016). The upregulation of MYC2 also highlights its involvement in both JA signaling and the MAPK pathway, further underscoring the crosstalk between these signaling networks (Xu *et al.*, 2019). Additionally, gibberellin (GA) signaling was activated in *A. tenella*, as evidenced by the

upregulation of gibberellin 3-beta-dioxygenase (GA3ox) and phytochrome-interacting factor 3 (PIF3), alongside the downregulation of the DELLA protein, a key growth repressor. This regulation suggests that *A. tenella* enhances GA biosynthesis to mitigate growth inhibition under Cr stress while balancing growth and defense responses (Colebrook *et al.*, 2014; Dobrikova *et al.*, 2017). Interestingly, the auxin (IAA) pathway displayed a more suppressed response under Cr stress. Although genes involved in IAA biosynthesis, particularly those in the Trp-to-IAA conversion pathway, were upregulated, downstream components of the auxin signaling cascade, including AUX1, ARF, and early auxin-responsive gene families like Aux/IAA, GH3, and SAUR, were largely downregulated. This suppression suggests that auxin transport and signaling were inhibited, leading to disrupted auxin-mediated growth and development processes (Hu *et al.*, 2013; Chen *et al.*, 2023). Previous studies have shown that Cd and Cr stress suppress auxin signaling by stabilizing AUX/IAA proteins and inhibiting auxin transport, a mechanism likely observed here (Yuan and Huang, 2016).

Other phytohormone pathways, including brassinosteroid (BR) and salicylic acid (SA), also exhibited significant alterations under Cr stress. In the BR signaling pathway, brassinosteroid receptor (BRI1) was downregulated, while its negative regulator BIN2 was upregulated, suggesting BR signaling inhibition. However, the upregulation of downstream transcription factors such as brassinosteroid-resistant 1/2 (BZR1/2) indicates a complex regulatory mechanism that balances BR-mediated growth responses under Cr stress (Hayat *et al.*, 2012). Additionally, salicylic acid-mediated defense pathways were activated, with pathogenesis-related protein 1 (PR1) showing upregulation, while non-expressor of pathogenesis-related genes 1 (NPR1) was downregulated. This likely reflects an SA-induced defense response aimed at enhancing plant resilience to Cr toxicity (Kohli *et al.*, 2017; Liu *et al.*, 2016).

Transcription factors (TFs) play a crucial role in regulating plant responses to HM stress, including Cr. In *A. tenella*, Cr(VI) stress triggered significant alterations in the expression of 2238 DEGs related to TFs, reflecting their central role in orchestrating

stress responses and metal tolerance mechanisms. The upregulated TF genes belonged predominantly to the WRKY, MYB, NAC, AP2/ERF, zinc finger protein families, and heat shock factors (HSFs), which have been previously identified as major regulators of HM stress responses. WRKY TFs are widely known for their involvement in abiotic and biotic stress responses. Under Cr stress, *A. tenella* exhibited upregulation of 270 WRKY TF genes, including WRKY75, WRKY21, and WRKY74. These WRKY members are well-documented for their roles in metal tolerance. For example, WRKY75 is associated with regulating stress tolerance pathways, including nutrient homeostasis and detoxification processes. Similarly, WRKY21 and WRKY74 are implicated in stress signaling cascades that activate antioxidant defense systems, contributing to the plant's ability to mitigate oxidative stress under Cr stress. These findings are consistent with previous reports where WRKY genes enhanced Cd tolerance in *Arabidopsis* by targeting specific metal transporter genes (Sheng *et al.*, 2019).

The AP2/ERF family, particularly its DREB subfamily, plays a significant role in abiotic stress tolerance by regulating dehydration and metal detoxification pathways. In *A. tenella*, 222 AP2/ERF TFs were significantly upregulated under Cr stress, with fold changes ($\log_2$ values) reaching up to 6.5 for some genes. Notably, the DREB proteins, including DREB2C and DREB2F, were significantly upregulated, highlighting their role in Cr stress response. DREB TFs are known to activate downstream genes involved in stress protection and ROS scavenging, thereby improving plant resilience to heavy metals (Xu *et al.*, 2019). The AP2/ERF subfamily's activation likely enhances stress signaling pathways and promotes the transcription of genes involved in antioxidant defense and metal chelation.

The MYB and NAC TF families also exhibited substantial upregulation under Cr stress, with 916 MYB-related DEGs and several NAC genes identified. MYB TFs are key regulators of HM stress, modulating pathways for detoxification, lignification, and ROS homeostasis (Hu *et al.*, 2017). Their upregulation in *A. tenella* under Cr stress suggests their involvement in strengthening cellular structures and promoting antioxidant mechanisms to alleviate Cr-induced oxidative damage.

Similarly, NAC TFs are known to regulate plant responses to abiotic stresses by modulating stress-related gene expression and promoting cell wall reinforcement. C2H2 zinc finger proteins are another major group of TFs implicated in heavy metal stress responses. In this study, 415 DEGs encoding C2H2 TFs were regulated under Cr stress. C2H2 zinc finger proteins are known to bind DNA and regulate the expression of genes involved in ROS detoxification, metal chelation, and stress adaptation. Their upregulation under Cr stress indicates their role in maintaining cellular homeostasis and mitigating oxidative damage caused by Cr.

Heat shock transcription factors (HSFs) were also significantly upregulated in response to Cr stress, with 174 DEGs encoding HSP proteins identified. HSFs regulate the expression of small heat shock proteins (sHSPs), which play critical roles in protein stabilization and prevent cellular damage under stress conditions. The upregulation of chloroplastic sHSPs suggests their involvement in protecting photosynthetic machinery and cellular proteins from Cr-induced oxidative stress. These findings align with previous studies where HSPs were induced under metal stress in switchgrass and cherry tomato (Su *et al.*, 2021; Song *et al.*, 2018). Cr stress also regulated the expression of other TF families, such as the bHLH family (378 DEGs), BES1/BZR1-like proteins, and TCP family. While most bHLH TFs were upregulated, BES1/BZR1 homologs involved in brassinosteroid signaling were significantly downregulated. Brassinosteroid signaling is critical for growth regulation and stress responses, and its suppression under Cr stress suggests a trade-off between growth and survival mechanisms. Additionally, the downregulation of TCP TFs, which regulate plant growth and development, further emphasizes the inhibitory effects of Cr on cellular metabolism and energy processes.

Cr stress disrupts both the light-dependent and carbon fixation phases of photosynthesis, reducing the plant's overall photosynthetic efficiency. Under Cr stress, the expression of unigenes encoding components of photosystem II (PSII) light-harvesting pigment complexes (Lhcb1-Lhcb7) was significantly downregulated. These complexes play a crucial role in capturing light energy and

transferring it to the reaction centers, and their reduced expression directly impairs light-harvesting capacity. Furthermore, most DEGs associated with PSII, including those encoding *psbK*, were also downregulated. This indicates that Cr stress diminishes PSII functionality, leading to impaired photochemical reactions and oxygen evolution. DEGs associated with photosystem I (PSI) and F-type ATPase were also downregulated. This suggests a compensatory mechanism to maintain ATP synthesis and energy transfer under Cr stress. Cytochrome b6/f complex, critical for electron transport, showed decreased DEG expression, indicating that the electron transport chain might be partially hindered. The mixed expression patterns of DEGs in the photosynthetic electron transport chain, with upregulation of some (e.g., *petF*, *petH*) and downregulation of others (e.g., *petE*, *petL*), reflect a complex response aiming to balance electron transport efficiency under Cr-induced oxidative stress.

The unique carbon assimilation pathway in *A. tenella* also exhibited notable adjustments. While the expression of phosphoenolpyruvate carboxylase (PEPC) and pyruvate orthophosphate dikinase (PPDK), enzymes central to C4 metabolism, increased significantly, the expression of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the primary enzyme for CO₂ fixation in the Calvin cycle, was reduced. This shift suggests a reliance on C4-like mechanisms to enhance photosynthetic efficiency and maintain energy production under Cr stress. The reduced Rubisco expression aligns with observations in other plants, where heavy metal stress preferentially suppresses C3 carbon fixation pathways. Furthermore, unigenes encoding the subunit IV, VA, and VIB of cytochrome C oxidase were significantly downregulated under Cr stress in *A. tenella*. Cytochrome C oxidase is essential for the mitochondrial electron transport chain and ATP synthesis. Its downregulation suggests that Cr stress inhibited the electron transport chain, thereby disrupting energy synthesis and exacerbating the energy deficit caused by impaired photosynthesis.

4.4.3. Tissue level expression of selected genes involved in Cr detoxification in *A. tenella*

The differential expression of selected genes in *A. tenella* under Cr stress showed a combined molecular response for metal detoxification, transport and stress mitigation. Each gene exhibited specific tissue- and time-specific patterns reflecting its functional role in Cr tolerance.

The upregulation of ABCC2 and YSL1 genes reflect the active mobilization and sequestration of metal ions in plant cells. YSL proteins are associated with the long-distance transport of metal-nicotianamine or metal-glutathione complexes. The strong induction of YSL1 gene expression especially in roots of *A. tenella* suggests the radial transport of Cr and its possible redistribution to the aerial tissues. YSL upregulation under metal stress including that imparted by Cd and Mn has been previously noted in *Medicago sativa*, *A. thaliana* and *O. sativa* (Zhang *et al.*, 2023; DiDonato Jr *et al.*, 2004; Sasaki *et al.*, 2011), supporting their involvement in metal homeostasis. ABCC2 is a member of the ATP-binding cassette transporter family which translocates glutathione-metal conjugates into vacuoles. While ABCC2 expression was transient in roots, its accumulation in leaves and stems increased progressively and peaked at later stages. This implies that ABCC2 may function in long distance transport and vacuolar sequestration of Cr-thiol complexes following chelation in the cytosol and thereby protecting photosynthetic tissues. Studies in *A. thaliana* and *O. sativa* have reported ABCC transporters that mediate Cd, Hg and As detoxification through vacuolar sequestration (Park *et al.*, 2012; Song *et al.*, 2014). The parallel expression pattern observed here indicates a similar protective mechanism against Cr in *A. tenella*.

The significant upregulation of PCS, GST and HIPP39 genes in roots and leaves implies their role in Cr detoxification through chelation and formation of complex. PCS is a key enzyme which catalyzes the synthesis of phytochelatins (PCs) from glutathione. PCs bind with Cr ions to form PC-Cr complexes and facilitate their sequestration into vacuoles. The strong induction of PCS genes especially in roots (up to six-fold) indicates an enhanced chelation to limit cytoplasmic metal toxicity.

The early induction in roots also indicates a primary role in metal binding at the uptake site, while expression in stem and leaves suggests chronic detoxification as Cr translocates. Earlier studies in *N. tabacum* and *O. sativa* supports these findings where PCS gene was upregulated under Cd and Cr exposure (Wu *et al.*, 2024; Nie *et al.*, 2021). The coordinated expression of PCS with ABCC2 signifies a regulated mechanism linking chelation and sequestration. Expression of GST also showed a high increase across tissues, consistent with its role in both detoxifying metals and antioxidant enzymes under heavy metal stress (Lin *et al.*, 2025). The enhanced expression in leaves implies the requirement of strong antioxidant defence in photosynthetic tissues as they are more prone to Cr-induced oxidative stress. GST-mediated detoxification has been reported in plants under heavy metals like Cd, As, Cu and Cr stress (Kumar and Trivedi, 2018; Nie *et al.*, 2021) and its upregulation in *A. tenella* aligns with the observed increase in glutathione concentration during Cr exposure. HIPP39 functions as metal chaperones and facilitates metal ion sequestration in plant cells. Its increased expression in roots during the early phase of Cr exposure is consistent with its role in binding and compartmentalizing Cr near the site of metal uptake (Barr *et al.*, 2023). Moderate increase in expression was also observed in stems and leaves although the response was delayed.

The differential regulation of XTH, LAC6 and CASP shows the detoxification responses in cell wall under Cr stress in *A. tenella*. XTHs remodel cell wall architecture to facilitate cell expansion and reinforcement under stress. Its expression declined in aerial tissues but was moderately induced in roots during early stress. This may imply reduced involvement of XTH in cell wall remodelling and strengthening under Cr stress as contrary to previous studies in *A. thaliana* and *Raphanus sativus* (Zheng *et al.*, 2021; Han *et al.*, 2025). Lignification of cell walls is a known mechanism for metal immobilization in apoplastic compartments and laccases (LAC) are oxidases involved in lignin biosynthesis. LAC6 showed strong upregulation in roots and downregulation in leaves and stems under Cr stress. The increased expression in roots is consistent with its known role in metal tolerance by restricting the movement of metal ions to shoots. But the downregulation in aerial parts suggests a reduced barrier formation against the Cr entry. Laccase mediated

lignification has been connected to enhanced tolerance to Cd and Cu in *A. thaliana* and *O. sativa* (Gaddam *et al.*, 2024; Liu *et al.*, 2017), which correlates with observed pattern in roots but contradicts in leaves and stems of *A. tenella* under Cr stress. CASPs are structural proteins critical for the formation of Casparian strips that functions as apoplastic barrier (Barbosa *et al.*, 2023). CASP showed an early induction in roots under Cr exposure that supports its involvement in reinforcing the endodermal barrier and limiting metal translocation to the vascular tissues. But the expression decreased in later stages of stress and in aerial tissues.

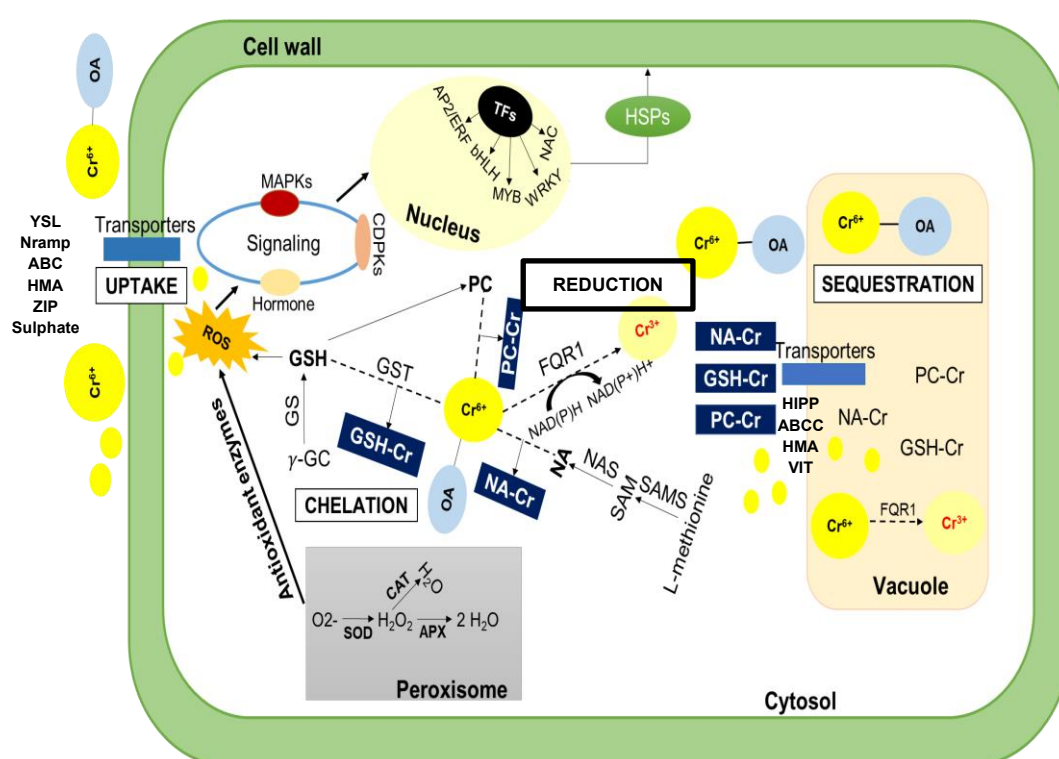


Fig. 4.17. A predicted regulatory model of molecular mechanisms involved in chromium detoxification in *A. tenella*.

A. tenella, thus employs a multilayered strategy to detoxify Cr and mitigate its toxicity (Fig. 4.17). The hierarchical activation pattern, chelation and sequestration in early stages followed by structural modifications and antioxidant defence reflects an adaptive mechanism to tolerate and detoxify excess Cr. Cr uptake is facilitated by specific transporters, while vacuolar sequestration and efflux mechanisms, supported by ABCC, MATE, and HIPPs, limit cytoplasmic Cr accumulation.

Enhanced biosynthesis of glutathione (GSH), phytochelatins (PCs), and metallothioneins (MTs), along with the activation of antioxidant enzymes like SOD, CAT, and the AsA-GSH cycle, help chelate Cr ions and manage oxidative stress. Dynamic cell wall modifications and the regulation of key transcription factors and signalling pathways, including MAPKs, calcium signalling and ABA, coordinate stress responses while suppressing growth related pathways. These integrated mechanisms highlight the plant's resilience to Cr stress and its potential for phytoremediation in contaminated environments.

Alternanthera tenella Colla (syn. *A. ficoidea* (L) Sm.) is an invasive alien plant belonging to the family Amaranthaceae and is known by vernacular names ranging from “Joseph’s coat plant” and “Joyweed” to “Kattu Ponnakanni” in Malayalam. In the present study, an attempt was made to identify a plant potential for phytoremediation and to analyse its responses and tolerance against selected heavy metals – cadmium, chromium and lead. A comparative study on different parameters in root, stem, and leaves of plants grown in Hoagland nutrient solution and subjected to known concentrations of these metals was performed to evaluate the impact of these heavy metals on growth, physiology, metabolic and molecular changes.

Phytosociological studies on the riparian vegetation in the Walayar Riverbank along the industrial area of Kanjikode was conducted using nested quadrat method and different diversity indices were calculated. *A. tenella* was observed to be the plant with highest IVI value and it showed heavy metal accumulation and translocation. The plants grown in half strength Hoagland medium without any metal treatment served as the control. Analyses of various functional and structural parameters in the root, stem and leaves were carried out on 7, 14, and 21 days of heavy metal treatment. For the standardization of heavy metal concentrations, rooted cuttings were grown in different concentrations of cadmium nitrate ($\text{Cd}(\text{NO}_3)_2$), potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$), and lead acetate ($\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$). The maximum concentrations of metal salts in which the rooted propagules of *A. tenella* survived but exhibited approximately 50% growth retardation were selected for the study. Hence, *A. tenella* plants treated with the standardized concentrations of heavy metal, i.e., cadmium nitrate (170 μM), potassium dichromate (240 μM), and lead acetate (680 μM) were used for the further analysis.

Morphological parameters like root length, shoot length and leaf area were calculated using graduated scale and graph paper. Tolerance index also was calculated and taken as a parameter to assess tolerance potential. Scanning electron microscopy (SEM) analysis was employed to observe the anatomical modifications in tissues of different plant parts. The SEM-EDX (SEM linked to Energy Dispersive

X-ray analysis) technique was also used to determine the changes in distribution pattern of elements under different heavy metal stress. Quantitative analyses of primary and secondary metabolites including pigments, protein, soluble sugar, proline, phenolics, and flavonoids were done. Since ROS production is triggered under heavy metal toxicity, MDA production and the function of antioxidant enzymes such as catalase, peroxidase and superoxide dismutase were also measured in different plant tissues. The interaction of metal ions with the functional group of biomolecules in the plant tissues were studied using FTIR. ICP-AES was used to study the bioaccumulation and bioconcentration of heavy metals in *A. tenella*. From all the morpho-anatomical and physiological analyses, chromium was found to have the most inhibitory effects on plant and the one to be phytoextracted. So, RNA-Seq and untargeted LC-MS/MS were employed to elucidate the molecular mechanism of chromium tolerance and detoxification in *A. tenella*.

The following conclusions were drawn after the analysis, comparison and discussion of the observed data using appropriate recent literature.

- The phytosociological study of the riparian vegetation Walayar Riverbank revealed a diverse angiospermic flora comprising 97 species under 32 families and 83 genera, predominantly represented by forbs and grasses.
- Poaceae and Asteraceae were the most speciose families, while therophytes dominated the life form spectrum reflecting adaptation to disturbed industrial environments.
- Diversity indices including Shannon-Wiener (2.192 to 3.143) and Simpson's dominance (0.855 to 0.947) indicated that the vegetation forms a diverse and stable natural community, though the contagious distribution of species suggested heterogeneity and dominance by a few taxa such as *A. tenella*, *A. sessilis*, *Sida acuta*, *Mimosa pudica*, and *Cynodon dactylon*.
- The dominance indicated by high IVI value and the high BAC and BTC values for Cr, Mn, And Zn showed *A. tenella* to be a resilient species capable of thriving

under industrial pollution stress and serving as a promising candidate for the phytoremediation of metal-contaminated riparian ecosystems.

- Heavy metal exposure caused significant inhibition of growth and visible stress symptoms in *A. tenella* and the toxicity increased in the order $Pb < Cd < Cr$. The retardation in the root and shoot growth reflected the metal-induced inhibition of cell division and elongation.
- Anatomical changes under metal stress included severe deformity in cell shape and structure, degeneration of parenchyma cells, increase in xylem diameter and reduced wall thickness, suggesting adaptive responses of the plant aimed at metal sequestration and stress tolerance.
- Pb accumulation was seen as electron-dense deposits in vacuoles, cell walls and intercellular spaces in roots and leaves of *A. tenella* which was confirmed by EDAX.
- Heavy metals induced partial or complete stomatal closure; guard cells were thickened under Cd stress. Trichome numbers reduced and stomatal density on the abaxial surface increased under all treatments.
- Heavy metal exposure restructured nutrient allocation and microelement homeostasis, replacing or displacing certain elements like the disappearance of Mg, Al, Si, Fe in roots and the appearance of Cl, Na, P, K, and Ca were redistributed.
- Cd and Cr treatments caused progressive reduction in shoot and root biomass, by 16-18% in shoots and up to 33% in roots at 21d compared to control. Pb stress had the least growth inhibition, hence the Pb-treated plants-maintained biomass levels compared to control.
- RWC decreased under all metal treatments with Cd causing the greatest decline (nearly 75% on 14d), showing early onset of dehydration and water deficit stress.

- Cr stress severely inhibited chlorophyll synthesis, as evident by the drastic reduction in chlorophyll a, chlorophyll b, and carotenoid content by 87%, 65%, and 22% respectively, at 21d. Pb-treated plants maintained comparative photosynthetic pigment levels, indicating better tolerance.
- Cd and Cr treatments induced early increase in protein accumulation followed by significant declines by 21d, indicating the synthesis of stress proteins in the early stages and the protein degradation or synthesis inhibition at prolonged exposure.
- Higher sugar accumulation suggesting an adaptive osmoprotective role was observed in Pb-treated tissues, while Cd and Cr treatments caused fluctuations in the soluble sugar level in all tissues.
- Accumulation of proline increased drastically under all heavy metal treatments in all plant tissues, with the highest levels observed in the leaves and stems. In leaves and stems, both Cd and Cr treatments caused a strong induction of proline by 13-16-fold, while Pb induced highest accumulation in roots by 8-fold.
- Biosynthesis of phenolics and flavonoids was strongly upregulated under metal stress, reinforcing their role in antioxidant defense and metal detoxification. Cr induced the strong enhancement of phenolic and flavonoid concentration.
- MDA content is an indicator of lipid peroxidation and oxidative damage. Cd and Cr exposure caused high increases in MDA levels, suggesting strong oxidative stress. The MDA accumulation was highest in roots under all treatments.
- *A. tenella* showed strong enzymatic antioxidant defense under metal stress, which was a tissue- and time-dependent variant. Cr and Cd induced more distinct responses than Pb, highest in roots and stems. CAT and POX showed more activity than SOD.

- GPX activity was significantly enhanced under Cd and Cr treatments in all tissues, but Pb exposure suppressed the enzyme activity. This suggests that GPX plays a crucial role against oxidative stress induced by Cd and Cr but is adversely affected under Pb toxicity.
- FTIR spectral shifts confirmed metal binding with hydroxyl, carboxyl, and amide functional groups highlighting the role of biomolecular interactions in detoxification. The 3200 - 3500 cm^{-1} region (O-H and N-H stretching) showed prominent changes indicating the interaction of metals with proteins, phenols, and polysaccharides.
- Cr-treated leaves displayed loss of absorption peaks in the 3200 - 3600 cm^{-1} and $< 900 \text{ cm}^{-1}$ regions that implies the selective binding of Cr to cell wall components. Pb-treated samples exhibited increased peak intensity in the 3200 - 3600 cm^{-1} region and disappearance of certain characteristic bands (1316 and 1241 cm^{-1}), indicating complexation of Pb with lignin and cellulose.
- Metal quantification showed maximum accumulation in roots, with the highest total uptake for Pb (46,866.9 mg kg^{-1} DW). The bioconcentration factors (19.04 for Pb; 8.39 for Cd) and low translocation factors (Cd - 0.505; Pb - 0.2) for Cd and Pb indicates that *A. tenella* preferentially retained Cd and Pb in roots, while Cr was more efficiently translocated to shoots (BCF - 16.23; TF - 1.03). These findings confirm the ability of *A. tenella* for phytostabilization of Cd and Pb and phytoextraction of Cr highlighting its adaptability for ecological restoration of metal-contaminated sites.
- Untargeted metabolomics of *A. tenella* leaves under Cr stress revealed the differential expression of 74 metabolites in which 26 were upregulated (36%) and 43 were downregulated (61%), indicating extensive metabolic reprogramming.
- Flavonoids were the most upregulated class reflecting a strong antioxidant response. Increased accumulation of glycosylated flavonoids like rutin and

kaempferol indicates enhanced carbon allocation toward ROS-scavenging metabolites and stabilization of phenolics under Cr toxicity.

- Altered expression of chlorophyll degradation intermediates such as pyropheophorbide a and pheophytin A showed the impaired photosynthetic pigment turnover under Cr stress.
- The downregulation of Caffeic acid and stilbene derivatives and lipid-derived metabolites like phosphatidylcholine suggests the stress-induced cell wall modifications which indicate the limited barrier formation to prevent Cr entry into the cells.
- RNA-Seq analysis of *A. tenella* leaves treated with Cr showed 5,94,917 transcripts and 63,085 unigenes with 1035 and 781 DEGs respectively of significant upregulation and downregulation.
- Transcriptomic profiling revealed the upregulation of genes involved in metal transport (like ABCC, HIPP, NRAMP, YSL), chelation and sequestration (PCS, GST, MT, NAS), reduction of Cr(VI) to Cr (III) (FQR), antioxidant defense and phytohormone signaling, while genes associated with cell wall modification (XTH, LAC, CASP, Expansins) and photosynthesis were largely downregulated. RT-PCR validation confirmed these transcriptional trends.
- Hence, the present research presents an integrated anatomical, physiological, and molecular strategy in *A. tenella* which contributes to its remarkable chromium tolerance.

Based on the current findings, some promising research directions are recommended to improve the ability of *Alternanthera tenella* for phytoremediation and to develop its efficiency for ecological restoration of heavy metal contaminated environments.

1. Microbial assisted phytoremediation

Since plant growth promoting rhizobacteria (PGPR) and mycorrhizal fungi can significantly influence the metal availability and physiological responses in plants, future research should explore the synergistic role of these microbes in supporting *A. tenella* during metal stress. These microorganisms contribute to plant health by improving nutrient uptake, regulating phytohormone levels, and producing siderophores or metal chelating compounds. Investigating the rhizospheric microbiome associated with *A. tenella* growing in heavy metal-contaminated areas and identifying beneficial microbial strains can help in formulating bioinoculants. Therefore, microbial-assisted phytoremediation can be made into a sustainable and scalable strategy using these symbiotic interactions that can significantly enhance metal uptake, tolerance, and accumulation.

2. Nanophytoremediation

The integration of nanotechnology with phytoremediation holds great potential to augment metal uptake and detoxification. Future research should focus on the use of green nanoparticles synthesized from *A. tenella* or other environmentally safe nanoparticles (e.g., silver oxide, zinc oxide or carbon-based) to enhance the bioavailability of heavy metals in the rhizosphere, either for *A. tenella* or other phytoremediating species. These nanoparticles may stimulate physiological and biochemical responses, thereby improving plant stress tolerance and antioxidant defense mechanisms. Comprehensive assessment of the phytotoxicity, absorption mechanisms, and long-term environmental impacts of nanoparticles is required to ensure safe and effective implementation of remediation strategies.

3. Constructed wetland systems

The profuse growth and heavy metal tolerance of *A. tenella* make it an excellent candidate for use in constructed wetlands to treat industrial effluents. Future research should prioritize designing and optimizing constructed wetland systems that include *A. tenella*, focusing on hydrology, substrate composition, and plant-soil-microbe interactions. These systems, combined with microbial fuel cell (MFC) technology, offer a novel approach for simultaneous wastewater treatment and bioelectricity production. The efficiency, energy output, and scalability of such hybrid systems need to be evaluated to develop sustainable and cost-effective remediation technologies.

4. Proteomics for functional characterization

Comprehensive proteomic analyses are recommended to identify and characterize proteins involved in heavy metal detoxification pathways, complementing the transcriptomic and metabolomic insights gained in the present study. Proteomics can reveal post-transcriptional regulation and identify novel stress-responsive proteins, since mRNA expression does not always correlate with protein abundance or activity. The key functional pathways and regulatory hubs involved in metal tolerance can be identified by integrating these omics datasets that can aid in the discovery of biomarkers and targets for genetic manipulation.

5. Genetic engineering and transgenic approaches

Genetic manipulation of *A. tenella* offers a potent approach to improve its phytoremediation capacity. Future research should focus on developing transgenic lines that overexpress genes involved in metal transport (e.g., YSLs, ABCs, HMAs), chelation (e.g., MTs, PCs, NA), and stress response (e.g., HSPs, antioxidant enzymes). Genome editing tools like CRISPR/Cas9 could be used to modify regulatory pathways and eliminate traits that limit metal accumulation. Bioengineered plants with higher metal uptake efficiency and adaptability to diverse contaminated sites can be produced by combining these molecular strategies with physiological and environmental insights.

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PRESENTATIONS AND PUBLICATIONS

Presentations

Firdous, K. A. & Resmi, M. S. (2025). "Transcriptional characterization of candidate genes related to chromium uptake, transport and accumulation in *Alternanthera tenella* Colla" at Kerala Science Congress on 8-10th February, organized by Kerala State Council for Science Technology and Environment at Kerala Agricultural University, Mannuthy, Kerala (Poster).

Firdous, K. A. & Resmi, M. S. (2024). "Physiological, anatomical and transcriptional responses of *Alternanthera tenella* Colla against chromium toxicity" at Kerala Science Congress on 9-11th February, organized by Kerala State Council for Science Technology and Environment at Government College, Kasaragod, Kerala (Poster).

Firdous, K. A. & Resmi, M. S. (2023). "Adaptive responses of *Alternanthera tenella* Colla to cadmium stress through physio-anatomical modifications and elemental distribution pattern" at National Seminar on Plant Biodiversity, Genetics, Breeding and Biotechnology on 7-8th December, organized by Department of Botany, University of Calicut (Oral).

Firdous, K. A. & Resmi, M. S. (2023). "Physio-anatomical modifications and mechanisms of tolerance against chromium toxicity in *Alternanthera tenella* Colla" at the Third International Scientific Conference on Environmental Research: Issues, Challenges and Strategies for Sustainable Development and Livelihood Security at Sub-regional Science Centre, Karwar, Karnataka on 1-2nd December (Oral).

Firdous, K. A. & Resmi, M. S. (2022). Heavy metal stress induced responses and mechanisms of plant defense in *Alternanthera tenella* Colla at the National Conference on Recent Trends in Plant Sciences and Biotechnology on 3-4th November, organized by School of Biological Sciences and Biotechnology (Botany), Goa University, Goa (Oral).

Firdous, K. A. & Resmi, M. S. (2022). "Comparative physiology combined with morpho-anatomical responses of *Alternanthera tenella* Colla to induced heavy metal stress" at the International Conference on Physiological and Molecular Mechanisms of Abiotic Stress Tolerance in Plants on 26-28th October, organized by Department of Botany, University of Calicut, Kerala (Oral).

Firdous, K. A. & Resmi, M. S. (2021). "Assessment of pollution tolerance and heavy metal accumulation in riparian flora around industrial area of Kanjikode, Palakkad" at the National seminar series on 25th September, organized by Research and PG Department of Botany, St. Thomas College, Thrissur (Oral).

Firdous, K. A. & Resmi, M. S. (2021). "Diversity of invasive plant species in the riparian zone of Walayar river in Palakkad district, Kerala" at the National webinar-DOCTRINA-17 on Trends in Plant Sciences on 10-12th August, organized by Department of PG and Research in Botany, Sir Syed College, Taliparamba (Oral).

Firdous, K. A. & Resmi, M. S. (2022). "Comparative physiology combined with morpho-anatomical responses of *Alternanthera tenella* Colla to induced heavy metal stress" at the International Conference on Physiological and Molecular Mechanisms of Abiotic Stress Tolerance in Plants on 26-28th October, organized by Department of Botany, University of Calicut, Kerala (Oral).

Publications

Firdous, K. A., Vivek, P. J., & Resmi, M. S. (2025). Growth, structural adaptations, and physiological dynamics of *Alternanthera tenella* Colla. toward lead toxicity. *International Journal of Phytoremediation*, 27(5), 700-710. <https://doi.org/10.1080/15226514.2024.2438768>

Kottakunnu Abdulrahman, F., Padmanabhan Jayanthikumari, V., Kizhekkepurath, N., & Mohankumar Saraladevi, R. (2024). Adaptive responses of *Alternanthera tenella* Colla. to cadmium stress through physiology, elemental allocation and morpho-anatomical modifications. *Acta Physiologiae Plantarum*, 46(7), 74. <https://doi.org/10.1007/s11738-024-03700-5>

Firdous, K. A., Vivek, P. J., Neethu, K., & Resmi, M. S. (2024). Physio-anatomical modifications and element allocation pattern in *Alternanthera tenella* Colla. associated with phytoextraction of chromium. *Environmental Science and Pollution Research*, 31(4), 5784-5806. <https://doi.org/10.1007/s11356-023-31597-z>

Firdous, K. A., Jidina, P., & Resmi, M. S. (2019). Diversity of invasive plant species in the riparian zone of Walayar river in Palakkad district, Kerala. *International Journal of Research and Analytical Reviews*, 6(4), 103-107. <http://www.ijrar.org/IJRAR1CEP017.pdf>

Workshops/Training

A hands-on journey to decode genomic and transcriptomic data, School of Biosciences, MG University, 2025.

Next generation sequencing, HelixOmics Analytics, 2024.

Next generation sequencing analysis, Decode Life, 2022.

Plagiarism, citations and proofreading, Research Graduate, 2022.

Research methodology, academic writing and publishing, Sir Syed College, Taliparamba, 2022.

Advanced techniques in molecular biology, medical biotechnology, industrial microbiology and bioinformatics, Amity University, 2021.

Tools and techniques in advanced plant science research, University of Kerala, 2019.

Research methodology, Department of Botany, SNGS College, Pattambi, 2019.