6. SUMMARY AND CONCLUSION

6.1 Bioaccumulation of heavy metals and physiological changes in mercury and lead treated plants

The exposure of Hg and Pb heavy metals for a short period induced a dose dependent physiological alterations as evidence by the biochemical changes, antioxidative enzyme changes, appearance and disappearance of DNA bands in the RAPD profile suggesting that the possible mechanism of Hg and Pb metal phytotoxicity in S. grandiflora and E. crassipes seedlings to be via oxidative stress. S. grandiflora and E. crassipes seedlings exposed to different concentrations of HgCl₂ (10, 20, 30, 40, 50, 60 mg L⁻¹) and Pb(NO₃)₂ (100, 200, 400, 600, 800, 1000 mg L⁻¹) exhibited inhibition of both root and shoot growth with shoots affected more than roots after 10 days of treatment. The reduction of Hg treated S. grandiflora seedling growth was 47% and 56% for shoots and roots respectively. Similarly, E. crassipes shoot and root growth was also reduced upto 60% and 61% at 50 mg L⁻¹ Hg levels, respectively. Growth reduction was positively correlated with Pb treatment and the maximum reduction of shoot growth noticed was 47%, while the root growth was decreased by 59%, compared to the control seedlings. Similarly, the E. crassipes shoot and root growth was reduced by 44% and 56% at 1000 L⁻¹ Pb levels. Most phytotoxic effects were observed at the highest dose of Hg (50 and 60 mg L⁻¹) and Pb (1000 mg L⁻¹) treatment. Apart from the reduction in growth, toxicity symptoms like chlorosis, drying of leaf edges, as withering, chlorosis, browning of roots, and dropped leaves were seen in higher Hg and Pb concentrations compared to control seedlings.

The total biomass was reduced by 19 and 35% in S. grandiflora and E. crassipes seedlings respectively, at higher concentration of Hg treatment with respect to the control. In Pb treated S. grandiflora and E. crassipes seedlings, the biomass was reduced by 21.40 and 33.28% with respect to the control seedlings.
The RWC was also decreased by 5% in S. grandiflora at 60 mg L\(^{-1}\) Hg treatment compared to control seedlings. However, E. crassipes did not show a significant change with mercury exposure levels when investigated. In Pb treatment S. grandiflora seedlings, RWC was decreased by 6.80% when compared to the control. However, E. crassipes increased slightly (3%) at higher concentration, with respect to the control.

The total chlorophyll and carotenoid contents in leaves of S. grandiflora seedlings were increased with increasing Hg and Pb dose compared to the control seedlings, though slightly decreased at higher concentrations. Seedlings treated with the 60 mg L\(^{-1}\) Hg dose showed 92% higher chlorophyll a, 76% higher chlorophyll b, and 69% higher carotenoid contents over the controls. In contrast E. crassipes seedlings, the level of photosynthetic pigments in leaves was decreased by 29.6%, 44.83% and 37.80% for chlorophyll a, b and carotenoid contents respectively at 50 L\(^{-1}\) Hg treatment and the reduction of chlorophyll a, b and carotenoid contents was 55%, 67% and 55% respectively, at 1000 mg L\(^{-1}\) Pb treated seedlings compared to the control. In this study, EDAX with SEM analysis revealed the presence of mercury and lead ion on both leaf and root tissues.

S. grandiflora and E. crassipes is a fast growing plant with high biomass production grown on hydroponics condition and has the ability to tolerate (60 mg L\(^{-1}\) HgCl\(_2\) for S. grandiflora and 50 mg L\(^{-1}\) HgCl\(_2\) for E. crassipes and 1000 mg L\(^{-1}\) Pb(NO\(_3\))\(_2\) for both seedlings grown up to 10 days) high mercury and lead concentrations. The level of mercury accumulation at 60 mg L\(^{-1}\) Hg treated S. grandiflora was 2.61 mg g\(^{-1}\) DW and 2.28 mg g\(^{-1}\) DW for root and shoot tissues respectively. In contrast, E. crassipes Hg content noticed was 2.04 mg g\(^{-1}\) dry weight in roots followed by 1.74 mg g\(^{-1}\) dry weight in leaf tissue at 50 mg L\(^{-1}\) Hg level. Accumulation of Pb content level was greater in roots than in shoots of both species at 1000 mg L\(^{-1}\) lead exposure level. In S. grandiflora seedlings, the shoots accumulated 23.06 mg g\(^{-1}\) dry weight, while roots accumulated 118.06 mg g\(^{-1}\) dry weight. In E. crassipes seedlings, the maximum accumulation of Pb content was 54.50 mg g\(^{-1}\) dry weight in roots and 06.60 mg g\(^{-1}\) dry weight leaf tissues. Results strongly showed that S. grandiflora and E. crassipes roots accumulated large
amount of mercury and lead than shoots. Further, S. grandiflora accumulated large amount of both heavy metals compared to E. crassipes. Therefore, the present study confirmed that according to our results, S. grandiflora seedlings not only capable to growing in the presence of high concentration of these metals but they are also able to incorporate them into their biomass and photosynthetic pigment contents. An efficient adaptation to hydroponics and the valuable Hg and Pb accumulation observed for S. grandiflora and E. crassipes plants, especially at higher doses of heavy metals, shows the great potential of this plant species for the decontamination of pollutants (mercury and lead) from the contaminated sites.

6.2 Effect of heavy metal stress on enzymatic and non-enzymatic antioxidants defense mechanism in mercury and lead treated plants

Mercury and lead heavy metal-induced cellular oxidative damage in plants has been shown to be due to excessive accumulation of reactive oxygen species (ROS), including free radicals and H₂O₂. To resist oxidative damage, the antioxidant enzymes and certain metabolites, including MDA content present in plants, play a vital role leading to adaptation and the ultimate survival of plants under stress conditions. Total soluble protein content was increased in S. grandiflora and E. crassipes seedlings based on the Hg and Pb treatment and slightly decreased at higher concentrations. The electrophoretic pattern of proteins of S. grandiflora and E. crassipes seedlings in response to treatment with different concentrations of mercury and lead after 10 days of treatment showed a variation in number of protein bands and intensity and/or density variations. Mercury treated S. grandiflora seedlings, the total MDA content observed was 60.25% and 58.45% increased for leaf and root tissues respectively, compared to the control seedlings. Maximum level of SOD activity noticed was 196% and 625% increase for root and leaf tissues respectively, CAT activity was increased about 16% and 66% for root and leaf tissues respectively, APX activity was increased to 28% and 34% for root and leaf tissues respectively and 67% and 35% of increase in POX activity was noticed in leaf and root tissues respectively, at 40 mg L⁻¹ Hg treatment than the control seedlings. The MDA content and all antioxidative enzymes such as SOD, CAT, APX and POX were increased upto 40 mg L⁻¹ Hg levels and then slightly decreased
at higher concentrations. In E. crassipes seedlings, maximum percent of MDA content noticed was 40% and 43% for leaf and root tissues respectively, compared to the control. SOD activity was increased 117% and 225% in root and leaf tissues respectively, CAT activity was also showed at 181.25% and 14.17% increase in root and leaf tissues respectively, APX activity was increased at 23% and 61% for root and leaf tissues respectively and POX activity was increased about 300% and 256% in leaf and root tissues respectively, compared to the control seedlings.

In case of lead treated S. grandiflora seedlings, maximum accumulation of MDA content noticed was 58% and 60% in leaf and root tissues respectively, compared to the control seedlings. Maximum level of SOD activity noticed was 677% and 274% increase in leaf and root tissues respectively and 76 and 148% increase CAT activity was noticed in leaf and root tissues respectively. The APX activity was significantly increased with increasing the Pb concentration up to 600 mg L⁻¹ in both the leaf (30%) and root (32%) tissues followed by slight decrease at higher doses and POX activity was increased to 82% and 100% in leaf and root samples respectively than the control seedlings and it was positively correlated with Pb treatment. In E. crassipes seedlings, maximum MDA content was 15% and 37% in leaf and root tissues respectively, compared to the control. Maximum SOD activity was 251% and 123% higher in leaf and root tissues of Pb doses respectively, catalase activity showed increases in leaf and root tissues up to 800 mg L⁻¹ Pb concentration (60% and 177% increment in leaf and root tissues), APX activity also showed 537% and 55% increases in leaf and root tissues respectively, and POX activity was increased about 589% and 254% in leaf and root tissues respectively, compared to the control seedlings.

In this study, Pb treated S. grandiflora seedlings, the presence of additional SOD isoform (SOD-V) could support the hypothesis that the SOD enzyme is being regulated strongly by Pb induced stress. CAT activity at higher doses was positively associated with a decreased intensity of CAT isoform in S. grandiflora. POX isozyme pattern showed that an additional POX isoform was appeared in Pb stressed tissues. The enhanced antioxidative enzyme mechanisms in S. grandiflora and E.
crassipes seedlings to heavy metal stress could help to overcome the metal toxicity by ROS detoxification.

6.3 Effect of heavy metal stress on DNA damage and genomic template stability by RAPD analysis

Heavy metals induced genotoxic effect on structure and function of genomic DNA molecule could be detected using molecular tools. Plant genomic DNA changes due to the exposure of genotoxic chemicals including heavy metal ions can be regarded as alterations in genomic template stability (GTS, a qualitative measure of genotoxic effect). The DNA was extracted from the leaf and roots of S. grandiflora and E. crassipes seedlings treated with Hg and Pb heavy metal ions along with untreated control and used for RAPD-PCR analysis. RAPD banding profiles showed clear differences between untreated control and Hg treated seedlings with distinct alterations (disappearance and/or appearance) in the number and size of DNA fragments with different primers. A total of 60 random oligonucleotide primers were screened for PCR amplification in which about 11 primers produced RAPD banding pattern and only five primers amplified clear, scorable and reproducible DNA fragments in S. grandiflora seedlings. The molecular size of the new DNA bands obtained with OPB-07 primer was a 500 bp, 1600 bp and 2600 bp in 10, 20 and 60 mg L\(^{-1}\) Hg treatments respectively, while a 450 bp DNA band was disappeared at 20 and 40 mg L\(^{-1}\) Hg exposed. In the case of OPB 10 primer, both 400 bp and 700 bp DNA bands were appeared in 10 and 20 mg L\(^{-1}\) Hg treatments respectively and those bands did not amplify in control leaf samples as well as in 40 and 60 mg L\(^{-1}\) Hg treatments. PCR amplification of leaf DNA with OPC 05, two DNA bands of 800 bp and 1000 bp were amplified in 20 and 40 mg L\(^{-1}\) Hg treatments respectively and 400 bp, 600 bp and 700 bp DNA bands were disappeared for 10, 20 and 40 mg L\(^{-1}\) Hg treatments respectively. In root samples, 800 bp, 1100 bp and 1200 bp DNA bands were appeared only at 40 mg L\(^{-1}\) Hg treatment and 300 bp, 400 bp and 700 bp DNA bands did not appear in 20 and 60 mg L\(^{-1}\) Hg treatments. The PCR amplification with OPC 09 primer, a 300 bp DNA band was amplified and 1100 bp and 1300 bp bands were missed in 10 and 20 mg L\(^{-1}\) Hg treatments compared to the untreated control leaf samples. With the primer
OPC 16, 250 bp and 300 bp DNA bands were amplified and 200 bp, 500 bp, 700 bp and 1000 bp DNA bands were disappeared at 10 and 20 mg L⁻¹ Hg treatments than control.

In Hg treated E. crassipes seedlings, a total of 60 10-mer oligonucleotide primers were screened for PCR analysis in which 20 primers generated RAPD banding pattern and only nine primers produced clear, scorible and reproducible DNA bands. Two newly amplified DNA bands were noticed with the RAPD pattern generated by OPA1 and OPA8 primers in the size of 1400 bp and 1700 bp respectively which were absent in the control. The PCR amplification with OPA16 primer with a 400 bp DNA band was amplified at 10 mg L⁻¹ Hg treatment. With the primer OPB1 generated RAPD pattern, two new DNA bands in the size of 1400 bp and 1300 bp band were obtained at 10 mg L⁻¹ Hg treatment. With the RAPD amplification profile of OPB11 primer, a 900 bp new DNA band was amplified at 10 mg L⁻¹ Hg treatment while a 500 bp normal DNA band was disappeared at 30 mg L⁻¹ Hg treatment. With OPB17 primer RAPD banding pattern, two additional DNA bands with 1500 bp and 1200 bp size were amplified at 10mg L⁻¹ Hg treatment. In the case of OPC9 primer based banding pattern, an additional DNA band (1700 bp) was amplified while a 1500 bp DNA band was disappeared at 30 mg L⁻¹ Hg treatment. In the OPC19 primer generated RAPD pattern, a 1600 bp band was appeared at 10 mg L⁻¹ Hg treatment which was absent in control as well as 30 mg L⁻¹ Hg treatment.

In S. grandiflora lead treated seedlings, a total of 60 10-mer oligonucleotide primers were screened for RAPD analysis in which 12 primers generated DNA profiles and only 4 primers produced clear, scorible and reproducible banding patterns. In the case of OPA10 primer, a 1200 bp DNA band was disappeared in all Pb treatments. The RAPD patterns of primer OPB1, a 900 bp DNA band was absent in all concentration except 600 mg L⁻¹ Pb treatment. A 500 bp band was appeared in all concentrations except at 1000 mg L⁻¹ Pb treatment and disappeared at higher concentrations in leaf samples. In contrast, a 500 bp DNA band was present in 400, 600 mg L⁻¹ Pb treatment when compared to the control root samples. The PCR amplification with OPB10 primer a 1000 bp DNA band was amplified only at 400
mg L\textsuperscript{-1} Pb treatment and disappeared in the remaining dose while a 700 bp band was disappeared in 200 and 400 mg L\textsuperscript{-1} Pb treatments. About 400 bp DNA band was present at 600 mg L\textsuperscript{-1} Pb treatment and absent in the remaining concentrations of roots samples when compared to the control. With the primer OPC16, a 700 bp DNA band was observed in all concentrations expect 200 and 800 mg L\textsuperscript{-1} Pb treatments, compared to that of control. Furthermore, the DNA changes detected by our RAPD assay could be used as a powerful molecular tool to identify the genotoxic effect of mercury and lead heavy metal toxicity induced stress in plants.

6.4 Synthesis and characterization of lead metal nanoparticles from heavy metal hyperaccumulator plants

Hyperaccumulator plants can accumulate exceptionally higher levels of heavy metals. In consideration of high metals concentrations in hyperaccumulator plants, the plants cannot be usually used as composting, agricultural forage and feed. The metals in plants must be removed, otherwise, they will return to and retain in soils. It is natural to come up with such imaginations using the Pb-hyperaccumulator plants as the material for synthesis of Pb metal nanoparticles. The concentration of lead in S. grandiflora shoots and roots were greater than 10,000 mg kg\textsuperscript{-1} (dry weight). The plant has a high tolerance for lead in soil, and S. grandiflora can be classified as a lead hyperaccumulator and used to remediate lead contaminated sites. Lead metal nanoparticles synthesized from S. grandiflora seedling and characterized by FTIR, XRD and SEM with EDS. The IR spectrum of lead nanoparticles obtained from this method showed the absorption peaks at 3278 represents N-H stretch (1 per N-H bond) amines group, 3186 represents N-H stretch amides group, 2921 represents C-H stretch Alkenes group, 1655 represents C=O stretch amides group, 1527 represents N-H bend (1°) amides group, 1426 denotes the O-H bend stretching carboxylic acids group, 1363 represents -NO\textsubscript{2} (aliphatic) nitro groups, 1243 represents C-O-C stretch dialkyl ethers group, 1102 represents C-C stretch ketones group, 1067 represents the C-N Stretch (alkyl) amines group, 540 cm\textsuperscript{-1} represents C-Cl stretch alkyl halides group. 3298 represents N-H stretch (1 per N-H bond) amines group, 3191 represents N-H stretch amides group, 2921 represents C-H stretch Alkenes group, 1649 represents C=O stretch amides group, 1527 represents N-H
bend (1°) amides group, 1423 denotes the O-H bend stretching carboxylic acids group, 1361 represents -NO₂ (aliphatic) nitro groups, 1103 represents C-C stretch ketones group, 1069 represents the C-N Stretch (alkyl) amines group, 536 cm⁻¹ represents C-Cl stretch alkyl halides group. FTIR results confirmed the presence of various phytochemicals viz., Phosphines, Sulfonates, Amides and Alky halides in lead treated of S. grandiflora. The presence of various phytochemical constituents in lead treated seedlings had reduced the lead ion into metallic lead nanoparticles compared to untreated control seedlings. The XRD pattern and EDX spectrum clearly showed that crystalline substances of the lead metal nanoparticles were synthesized. The synthesized nanoparticles were crystalline in nature. Furthermore, the diffraction peaks were few to be narrow, which showed the crystalline nature of lead metal nanoparticles. The presence of very short and broad peaks indicates that the synthesized lead metal nanoparticle was amorphous. In addition, the morphology of most synthesized lead metal nanoparticles is sphere shaped with a mean size of 13-60 nm in leaf and 30-61 nm in root tissues. These results indicated that S. grandiflora plant possess the great potential for lead accumulation and nanoparticle synthesis and also shows promising results for the development of a “green” process for nanoparticle synthesis using this fast growing S. grandiflora plant.

Results strongly showed that S. grandiflora and E. crassipes roots accumulated large amount of mercury and lead than shoots. Further, S. grandiflora accumulated large amount of both heavy metals compared to E. crassipes. In E. crassipes, transfer of both metals from roots to shoots was higher than S. grandiflora. Therefore, the present study confirmed that most phytotoxic effects were observed at the highest dose of Hg (50 and 60 mg L⁻¹) and Pb (1000 mg L⁻¹) treatment.

The enhanced antioxidative enzyme mechanisms in Sesbania and Eichhornia seedlings to heavy metal stress could help to overcome the metal toxicity by ROS detoxification. Thus the plants have efficient detoxification strategy to scavenge excess of ROS very efficiently by activation of SOD, CAT, APX and POX, which were regulated together in a coordinated way. These results clearly suggest that S. grandiflora and E. crassipes were able to tolerate higher concentration of Hg and Pb
toxicity and could hyperaccumulate a significant amount of Hg and Pb content in roots than in shoot tissues.

DNA changes detected by RAPD technique could be used as a powerful molecular tool to identify the genotoxic effect of heavy metal stress in plants. The DNA alterations in the RAPD profiles were found in both leaf and root tissues of S. grandiflora and E. crassipes seedlings. Therefore, S. grandiflora and E. crassipes had an effective molecular mechanism to survive under heavy metal stress by making alterations at genome level.

Lead accumulation level was found to be higher in root than shoot tissues of S. grandiflora seedlings hydroponically treated with 1000 mg L$^{-1}$ lead nitrate. Lead nanoparticles were characterized by FTIR, XRD and SEM with EDS. FTIR results confirmed the presence of various phytochemicals viz., phosphines, sulfonates, amides and alky halides in the leaf extracts of S. grandiflora. The XRD pattern clearly showed that crystalline substances of the lead metal nanoparticles were synthesized. The morphology of the lead nanoparticles was observed as spherical in shape. In addition, the SEM images demonstrated that lead metal nanoparticles were polydispersed and not uniformly distributed. Lead metal nanoparticle synthesis from metal hyperaccumulator plants may also provide a new route for the recycling of metals in a plant sources.