SUMMARY

The present research work embodied some of the important and salient features of Marsilea minuta L., an aquatic water fern which is exposed to elevated concentrations of Cd. This is more exercised with physiological and cellular responses when this fern species interacted in varying ways with polyamine under Cd concentrations. Taking altogether, the present thesis summarizes that Marsilea plants responded well in connection with metal toxicity as well as proved to be an efficient hyperaccumulating species. Moreover, the varying concentrations of Cd (0, 50, 100 and 200 µM of Cd) has proportionately induced the plant’s physiological activities in such a way those could be taken as biomarkers. Not only that, the interaction with polyamine (2 mM spermidine) has also modulated of plant’s response in Cd toxicity in such a way that could be hypothesized that polyamine may similarly be effective in modulation aspect for non-flowering plants. Finally, the behaviour of Marsilea plant in interaction with Cd has strongly evoked the scope for hyperaccumulation property of this plant.

From the preliminary physiological point of view, the plants were evaluated on their growth status out of Cd hyperaccumulation studies both by tissue staining method as well as spectrophotometric analysis. Initially, it was recorded that Cd offered its effect more critically on the plant’s water relation and vigour. For evaluating the osmotic balances under Cd treatments for prolonged seven days, the root length was significantly decreased linearly as a function of Cd doses. The dry matter accumulation by root was again retrieved partially by spermidine (Spd) treatment. On the aerial shoot part, the signaling of Cd accumulation was reflected both in petiole length and chlorophyll content of leaflets. The dry matter accumulation by shoot portion was subdued by Cd hypersensitivity was also reflected in relative growth rate (RGR) and specific leaf weight (SLW). For both the cases, the plants had minimized their curtailing of growth with Spd application. In cellular level, a significant accumulation of Cd was more into the
cortical region extending to the vascular conducts which were the features to be distorted as a function of Cd doses. Application of Spd had minimized this tissue damages owing to concurrent lesser metal accumulation. On close analysis with FTIR spectra, plants were characterized regardless of Cd concentrations in respect to changes of cell wall residues. The possible interacting functional groups for Cd binding affinity were monitored as alkyl halide (-C-Cl) within 770-772 cm\(^{-1}\). The most affected cell wall residues were demarcated as –COOH and –C=O groups of carbohydrate and protein residues. Significant alteration was also recorded for Spd interaction on those functional groups possibly indicating the mitigation of Cd induced any torsion of the cell wall.

Along with the metal accumulation in abundance, the *Marsilea* plant had recorded some changes in water relation. It is the relative water content (RWC) that recorded in decreasing manner indicating the plants are under water deficit. On close analysis to impart osmotic support, plants experienced with significant amount of osmolyte accumulation like proline and glycine betaine. The former was also concomitantly recorded with two rate-limiting enzymes like γ-GK and γ-GPR in its biosynthesis. For both the osmolytes, Spd had been evident as a good reliever to recover the osmotic balance to increased activities of γ-GK and γ-GPR. As a secondary effect of Cd stress, plants have experienced an oxidative exposure that has dismantled almost all of its cellular integrity. Cd being a potent prooxidant, has left hardly any biomolecules to be impaired including protein and lipid.

Contextually, the most primary effect of ROS is mediated by superoxide (O\(_2^−\)) radical and H\(_2\)O\(_2\) content in plant metabolism. A linear rise of those two undoubtedly recorded that *Marsilea* plants were under exposure of oxidative stress. The secondary effect of ROS comes through hydroxyl radical (OH\(^{−}\)) generation and its concomitant biomolecule disintegration. Thereby, OH\(^{−}\) radical content was accumulated in excess manner all though the Cd doses and thereafter minimized by Spd application. As biological consequence of ROS, lipid peroxidation and protein oxidation are granted as most reliable sign in plant system. Thus, above mentioned two biochemical phenomena in terms of MDA content and carbonyl content respectively exceeded several fold over the control and thereafter minimized with Spd application. Plant’s metabolism with regards to stress induced metabolites is a reliable index for assessment of physiological changes. So, when polyamine profiles were concerned in *Marsilea* plant under Cd induction, there recorded a linear increase in total pool of polyamine. This was further recorded that the highest concentration of polyamine though recorded maximum at 200 µM of Cd, but hardly any significant changes with respect to Spd application. With the ongoing concentrations of Cd, the more accumulated polyamines were apportioned into conjugated forms rather than soluble ones. This may refer that Cd toxicity might have induced a major amount of polyamine pool in complexation with other moieties. Therefore, the TLC and HPLC separated polyamine fractions needs to be quantified to detect...
any of special polyamine and their effective concentration if there. Then it is found that Spd was the major accumulated fraction amongst other fractions of polyamines.

In general aspects, Cd toxicity in plants may alter the nutrient balance that interferes the normal mode of metabolism. Undoubtedly, nitrogen being the most abundant and important nutrient in plant’s growth and development was also analyzed in *Marsilea* plants in the present experiment. It was mainly dealt with the major nitrogen metabolizing enzymes in conversion of inorganic nitrogen to organic residues. Initially, it was found that nitrate reductase (NR) both in root and shoot were found to be affected by a decreasing trend. This fall in enzyme activity was recorded to be maximum under 200 µM of Cd but retrieved with Spd in similar proportion of shoot and root. The next enzyme in succession is glutamate dehydrogenase (GDH) with its requirement of NADH and NAD(P)H. GDH (NADH linked) in shoot and root were discriminating in nature by down and up regulation respectively. The trend got reversed for both the enzymes with Spd application. On the contrary, NAD(P)H linked GDH in shoot and root almost recorded a similar trend being maximally curtailed at 200 µM of Cd over the control (0 µM) set. Spd was able to retrieve the activity of NAD(P)H linked GDH both in shoot and root and thus proved its efficacy to synthesize glutamine. Glutamine synthetase which reversibly converts glutamic acid to glutamine has downregulated the activities under Cd stress more with shoot than root. This refers the probability of amino nitrogen into amine nitrogen fractions in shoot. Finally, the interconversion of glutamine to 2-oxoglutarate was the focus to investigate the pool of glutamic acid under Cd stress. It was found that more glutamine remains in the cell rather than maintaining a steady conversion into glutamic acid in both shoot and root. This was monitored by the activity of GOGAT in shoot and root in an interesting trend that plants had maximized the activity at 50 µM and thereafter a steady decline. This may be indicative for the threshold values of GOGAT activities within 50 µM of Cd concentration. In a similar way, the activity was replenished with Spd application. Therefore, *Marsilea* plant responded in differential way to interact nitrogen assimilation under Cd contamination.

As in case of higher plants, the photosynthetic reserves are fractionated into different sugar profiles, mostly in structural and storage compounds. *Marsilea* plant being an aquatic in nature, only the ministructures of leaflets are offered for solar energy utilization. Thus, the downstream reactions of carbon metabolism from reduced carbon residues are apportioned into different complex moieties and there changes under Cd toxicity may describe the photosynthetic activities. Thus, the total carbohydrate in the form of both starch and reducing sugars recorded to be decreased in a linear fashion under Cd stress. The downregulatory trend of total carbohydrate may otherwise indicate the partial subdued photosynthetic activities under Cd toxicity. The retrieval with Spd for carbohydrate fractions possibly has arisen the gas exchange through foliages and/or in carbon assimilation reactions. On account of storage carbohydrate,
plants are also distinctly variable in their accumulation and abundance. Therefore, the most important cell wall constituting polysachharides are cellulose and hemicellulose which were significantly accumulated in the tissue under Cd concentration. This might be granted as one of the thickening phenomenon of metal induced cell wall modification. The interesting feature is that the accumulation of hemicellulose is more abundant than cellulose, however, both were maximum at 200 µM of Cd concentration. The Spd on its activities had moderated the cellulose and hemicellulose abundance over the cell wall and thereby had arisen the possibility for minimization of metal induced exertion on the cell wall. Interestingly, sugar metabolizing enzymes, particularly, sucrose hydrolyzers: soluble invertase and cell wall bound invertase recorded a declining trend all through the Cd concentrations. This is interesting to note that downregulation of sucrose hydrolysis might be restricted for more transportable products in growing shoots where metals are less abundant. Another possibility might be arranged with Cd sensitivity on those enzymes to delimit their activities of Spd in both the cases that had been able to erase the toxicity of Cd and evoked activities. On the other hand, starch degrading enzymes, particularly, α and β isoforms recorded a declining trend of the Marsilea shoot with Cd exposure. The downregulation of starch hydrolysis could bring the possibilities in favour of more polysaccharide accumulation, however, for mechanical support in the cell wall. Still, Spd had minimized the effects of Cd on amylases and relieved the activities in accordance with more starch hydrolysis. In a different mode, metal may induce some sort of restrictions in normal glycolytic pathway and thereby plants are to adopt anaerobic respiration. The alcohol dehydrogenase (ADH) activity in total plant tissues of Marsila plant were significantly induced all through the Cd concentrations. This satisfies the thoughts for plant’s preference for alcoholic fermentation with more ADH activity. When the plants were treated with Spd, the maximum effect on ADH activity in curtailing was reduced significantly. Organic acid metabolism precisely which is derived from central anaploretic pathway, like TCA cycle was also focused in the present experiment to observe the Cd toxicity, otherwise. Thus, malate synthesis in a reversible conversion with oxaloacetate by NAD(P)H/NADH linked malate dehydrogenase was found to be inhibited. Thus, Marsilea plant may appear in economic mode to minimize the reducing equivalents (NAD(P)H/NADH) in reduction of oxaloacetate to malate. The decrease in activity of malate dehydrogenase was also compensated by Spd that was observed with its upregulatory mode.

In association with Cd mediated oxidative stress, plants were well responded in fluorescence microscopic method for detection of ROS. It is the Evan’s Blue staining and thereby the realization of fluorescence indicated tissue dissolutions out of free radicals. Thus, a significant colouration of Evan’s Blue staining detected the specific zones from the root tip extending upwards as most sensitive. In Marsilea plant the features of roots under Cd stress might be a clear indicative feature for oxidative stress.
In connection to signaling of the plants for oxidative burst, a number of elicitors have undergone in nature which need to be cited. *Marsilea*, an aquatic fern, is not an exception of those where a clear picture of nitric oxide (NO) mediated signaling was scored. In accordance to Cd concentrations, the cut petiole section revealing the vascularization developed a distinct bluish green fluorescence. This fluorescence is typified for NO signaling in plants which has undergone moderated with Cd toxicity.

It is well established from the present experiment so far that the *Marsilea* plants are undoubtedly under oxidative stress out of Cd toxicity. Therefore, in accordance with hyperaccumulation of Cd and to accumulate the oxidative stress in such a way that plants could be affected minimally. Therefore, the stable and constitutively expressed antioxidants include the phenolics which are well regulated in *Marsilea* plants also. A significant upregulation of total phenolics has characterized the *Marsilea* plant to induce non-enzymatic antioxidative pathway. A steeper rise in total phenolic content was recorded in *Marsilea* plant tissues consistently as a function of Cd concentrations. Thus, the Spd treatment also reduced their abundance and implied the take-over charges by polyamine for phenolics. In phenylpropanid pathway for phenolics, it is the phenylalanine ammonia lyase (PAL) has experienced an upregulatory trend over the Cd doses. This undoubtedly advocated the regulation of phenolic biosynthesis under Cd hyperaccumulation to minimize the oxidative stress. The involvement of PAL is more evident from its downregulatory nature with Spd treatment at the highest concentration of Cd dose (200 µM). It is interesting to note that though *Marsilea* plants were induced to overexpress the total phenolics, however, the fractions of each residue was variable. It is more significant to focus the individual residues like ferulic acid, p-coumaric acid and caffeic acid for their accumulating trend in different modes. There was a consistent increase of p-coumaric acid and caffeic acid content while a decline of ferulic acid residues. This showed that individual residues of phenolic acids might be played with different manner in Cd tolerance with reference to antioxidation.

Along with evocation of non-enzymatic antioxidative pathway, plants are also tuned with some gene expressions which are often linked with some enzymatic cascades. Those enzymes are required to bring down the oxidizing level of ROS into different moieties and finally to water (H₂O). The gene expression with regards to enzymatic antioxidative responses are well coordinated into different profiles in the present experiment with *Marsilea* plants those have characterized as a few biomarkers adhered to oxidative stress. Both *in vitro* and *in gel* analysis of those enzymes were carried out with the background of Cd hyperaccumulation of *Marsilea* plant. Initially plants recorded a significant upregulation of superoxide dismutase (SOD) for O₂⁻ into H₂O₂ and H₂O and recorded a significant upregulation. This was followed by upregulation of other enzymes in respect to lysis of H₂O₂ in over expression manner. Thus, the guaiacol peroxidase (GPX), ascorbate peroxidase (APX) were recorded a linear rise with peak at 200
µM of Cd concentration following relieved with 2 mM Spd. Along with in vitro activities, plants showed a distinct and variable polymorphisms of those enzymes when studied in gel activities. As compared to control set (0 µM), the demarcation of specific band numbers and intensities have characterized those enzymes in stipulated manner. Catalase (CAT), the only enzyme showed on the contrary, a diminishing trend in their activities all through the Cd concentrations. The retrieval of activities by Spd has undoubtedly demanded the efficacy of polyamine in retrieval of the enzyme protein turnover by ROS. For the interconversion of reduced glutathione (GSH) in maintenance of cellular redox, plants have modulated their efficiencies by the supplementation of Spd under Cd treatments. A distinct variation both in band numbers and intensities are more supportive for their redox stabilization by GR activities as good as angiospermic plants. Structural integrity under metal stress through strengthening the cell wall by more lignification has also being monitored with cell wall bound peroxidase (WPOD) in Marsilea plants. A peak increase of WPOD from minimum to maximum Cd doses established the efficacy of peroxidase for lignifications. The role of Spd on recovery of WPOD in Marsilea plant has also been discussed.

Marsilea minuta L., is an aquatic herb which is sensitive under Cd stress has been evident from earlier communications. Depending on the availability of the facts and figures, it becomes prudent to justify the in vitro efficacy of antioxidation potential of the species. It has been also relevant to summarize the effects of Cd on such in vitro antioxidation properties along with the changes of other physiological attributes. Thus starting from DPPH radical scavenging to ABTS radical scavenging, plants recorded a significant increase along with the Cd concentrations accumulated in their plant tissue. This has also been relevant from their percent of inhibition taking different chemical analogues inducing oxidative stress by ROS generation. Spd, in both the cases, appeared as moderately effective in relieving the percent inhibition with those substances. On the contrary, Fe$^{2+}$ chelation method has appeared in a decreasing trend as a function of varying Cd concentrations. This undoubtedly showed that Marsilea can induce the chelation mechanism with some phytochemicals that sequester the metal in an operative way limiting the extent of damages of biomolecules. Spd, in this case also appeared as a successful moderator in increasing the Fe$^{2+}$ chelation with synthetic compound as EDTA. In another way, Marsilea plant was evident to be more potential in scavenging of OH$^-$ radicals. This got implemented with HOCl and OH radical scavenging assay by the compounds named ascorbic acid and mannitol respectively. Regardless of OH$^-$ radical scavenging assay, plants recorded a significant upregulation by several folds. Polyamine could be replenishing in reducing the radical scavenging potential in vitro and thus established its role in diminishing of OH$^-$ and H$_2$O$_2$ scavenging. Though these two moieties not regarded as typical ROS, still, those behaved to be disintegrating in the Marsilea tissue. The fractions of ethanolic/organic extract applied for H$_2$O$_2$ radical scavenging detection was evident to be upregulated. Similarly, Spd has taken the
substituting role when supplemented with extract of highest concentration and moderating the radical scavenging activity. Non-protein thiol with different molecular configurations and extended sulphohydryl (-SH) groups required to reduce the ROS in different extent. Collectively, this property is offered as reducing power activity and regarded as plant potential to nullify the effect of ROS, however, in direct way. *Marsilea* plant emerged as more potential in induction of reducing power activity in accordance with elevation of Cd concentrations. Reducing power activity was found to be moderated when Spd was applied along with highest dose of Cd in the *in vitro* extract. Therefore, these phenomena establish free radical scavenging activity of *Marsilea* plant along with ameliorating role of Spd there on this plant.

The photosynthetic performances and its characterizations are generally attributed both by light harvesting-energy transduction and carboxylation-reduction mechanism in plants regardless of taxonomic hierarchy. *Marsilea* being an aquatic fern species to be vulnerable for photosynthetic impairment under metal stress, definitely on to major domains as stated above. Thus, a significant change in transcript accumulation for photosystem II (PS II) was recorded. On the contrary, as a function of Cd doses, Rubisco larger subunit (rbc L) genes were remained insensitive. Spd application though had a significant modulation on growth parameters through dry matter accumulation but PS II and rbc L had hardly any response with Spd under Cd toxicity. Therefore, *Marsilea* plant showed its irreversibility in damages of photosynthetic mechanisms under Cd interference with any chemical elicitors like polyamine.