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Heavy Metal-Induced Protein Changes in *Amaranthus spinosus* L.: A comparative SDS- PAGE Study of Roots and Leaves

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Abstract

Research on protein profiling in plants exposed to heavy metal pollution is essential for understanding how plants react to harmful metals like lead, cadmium, and mercury. Plants exposed to heavy metals exhibit markedly altered protein profiles, with some proteins being repressed and others overexpressed. In order to deal with metal toxicity, these modifications aid plants in developing tolerance mechanisms. In this study, SDS-PAGE analysis is used to examine the effects of heavy metal stress (Pb, Cd, and Cr) on protein profiles in *Amaranthus spinosus*. The findings demonstrate observable alterations in electrophoretic protein bands, with metal-treated plants exhibiting the emergence of new bands and the elimination of pre-existing ones in contrast to controls. Heat Shock Proteins (HSPs) are important in plant defense systems, and the study reveals certain polypeptides linked to heavy metal stress tolerance. This study shows that heavy metal stress (Pb, Cd, and Cr) dramatically changes the protein profiles of *Amaranthus spinosus*, causing some stress-related polypeptides, such as HSPs, to be expressed while suppressing others. The plant's defenses against metal poisoning are demonstrated by the observed alterations in protein band patterns. These results highlight the potential of protein profiling as a method for comprehending plant tolerance to heavy metals.

Keywords: Heavy metal toxicity, SDS-PAGE, Electrophoretic protein bands, Heat Shock Proteins (HSPs), Stress tolerance, Environmental biotechnology

Introduction

Since the completion of genomic sequencing in many plant species, scientists have faced a big challenge of designating the role and function of each gene and their products (Sharma et al., 2008). To obtain deep insight of plant response to stress condition, traditional biochemical and physiological approaches were no longer sufficient (Ashan et al., 2009). So plants respond to abiotic stress by the changes in the protein expression. The response is accompanied not only by an alternation in the gene expression but also by qualitative and quantitative changes in proteins. Gel electrophoresis is the most commonly used technique to provide protein separation and this method was on the basis of migration of charged molecules in an electric field in a gel medium. The common

separation techniques employed in the analysis of proteins were one and two dimensional PAGE methods. Both these techniques are employed to profile cell or tissue proteins or to determine the purity of proteins (Klose, 2009). The two dimensions (2-D) is a classical SDS-PAGE which separates protein according to their molecular weight. Thus thousands of proteins can be separated by SDS PAGE and the information such as molecular weight, isoelectric point (pI) and the amount of each protein can be obtained (Friedman et al., 2009).

Today, the application of proteomic studies is expanding rapidly in the field of heavy metal stress biology and it is mainly focused on the functional translated portion of the genome. However, proteomic studies of metal stress have been far less numerous and comprehensive in comparison with investigations regarding other environmental stresses in plants (Hossain, 2013). Although the mechanism of heavy metal uptake, accumulation, distribution, oxidative stress, induction and detoxification have been investigated in a wide range of studies on plants (Cuypers et al., 2001; Aravind & Prasad, 2003; Gratao et al., 2008), the mechanisms involved are still only partially understood

The proteomic approach can therefore help elucidate new aspects of plant metal stress. In recent years, due to the advancement in techniques involving the identification and separation of proteins, substantial effort has been made in the clarification of molecular mechanisms underlying heavy metal toxicity in plants. However, the importance of protein profiling is still relevant. Number of proteomic studies that have been carried out under different stress conditions in plants is relatively low, but for heavy metal induced stress, proteomic studies are considerably even less represented (Liao et al., 2005). This methodology is most frequently applied in studies dealing with heavy metal stress in plants. It is based on the comparison of different proteomes, which originate from non-stressed plants and corresponding plants exposed to heavy metals (Kieffer et al., 2008). There are so many reports on the SDS PAGE analysis of proteins in plants exposed to heavy metals (Jomova & Morovic, 2009; Stefanic et al., 2012; Rout & Sahoo, 2012; Gomes et al., 2012; Aldoobie & Beltagi, 2013).

Material and methods

A weedy species, *Amaranthus spinosus* luxuriantly distributed on the road sides and abandoned land of Kerala was selected for the present study. The plant was recognized, and it was placed in the Kerala University Botanical Herbarium under voucher number KUBH 6032. For performing the protein profiling, the leaves and roots were collected from 60 days old heavy metal treated (Pb- lead 100, 150 and 200 µM, Cd- 5, 10 and 15µM and Cr-100, 150 and 200 µM) and control plants were collected. After collection, the plant parts were thoroughly washed with tap water to remove the soil particles or any other debris. SDS PAGE is a rapid method for quantifying, comparing and characterizing proteins. SDS PAGE method separates proteins on the basis of their molecular weight. SDS binds along with the polypeptide chain and the length of the reduced SDS protein complex is proportional to its molecular weight.

The reagents used included a 30% acrylamide stock solution (29 g acrylamide and 1 g Bisacrylamide in 100 ml distilled water), 1.875 M Tris HCl separating gel buffer (pH 8.8) and 0.6 M Tris HCl stacking gel buffer (pH 6.8). Polymerizing

agents consisted of 0.5 g Ammonium Per Sulphate (APS) in 10 ml distilled water and TEMED. For sample preparation, 0.5 g of fresh plant tissue was homogenized with 2 ml of 0.2 M phosphate buffer (pH 7) and centrifuged at 3,000 rpm for 5 minutes. A 12% resolving gel was prepared with 6.6 ml distilled water, 8 ml 30% acrylamide, 5 ml Tris (pH 8.8), 0.2 ml SDS, 0.2 ml APS and 8 µl TEMED, while a 5% stacking gel consisted of 4.6 ml distilled water, 1.7 ml 30% acrylamide, 2 ml Tris (pH 6.8), 0.1 ml SDS, 50 µl APS, and 10 µl TEMED.

The electrode buffer contained 6 g Tris, 14.4 g glycine and 1 g SDS in 1 litre distilled water (pH 8.2-8.4). SDS gel loading buffer was prepared with 85 µl Bromophenol Blue and 15 µl â-Mercaptoethanol. The gel was cast, and samples were loaded with 50 µl sample and 25 µl dye. Gel running was done at 100 volts, and the gel was stained with Coomassie brilliant blue R 250 solution and then destained. Molecular weight and Rf values were calculated using Labimage Platform software after documentation using a Transilluminator, Biotech R&D Laboratories, Yercaud gel imager.

Results and discussion

SDS- PAGE analysis of heavy metal treated root and leaf samples of *A. spinosus* exhibited visible changes in electrophoretic protein bands (Figs. 1, 2; Tables 1, 2). Significant changes were noticed in the protein pattern of control and treated samples. The root samples of

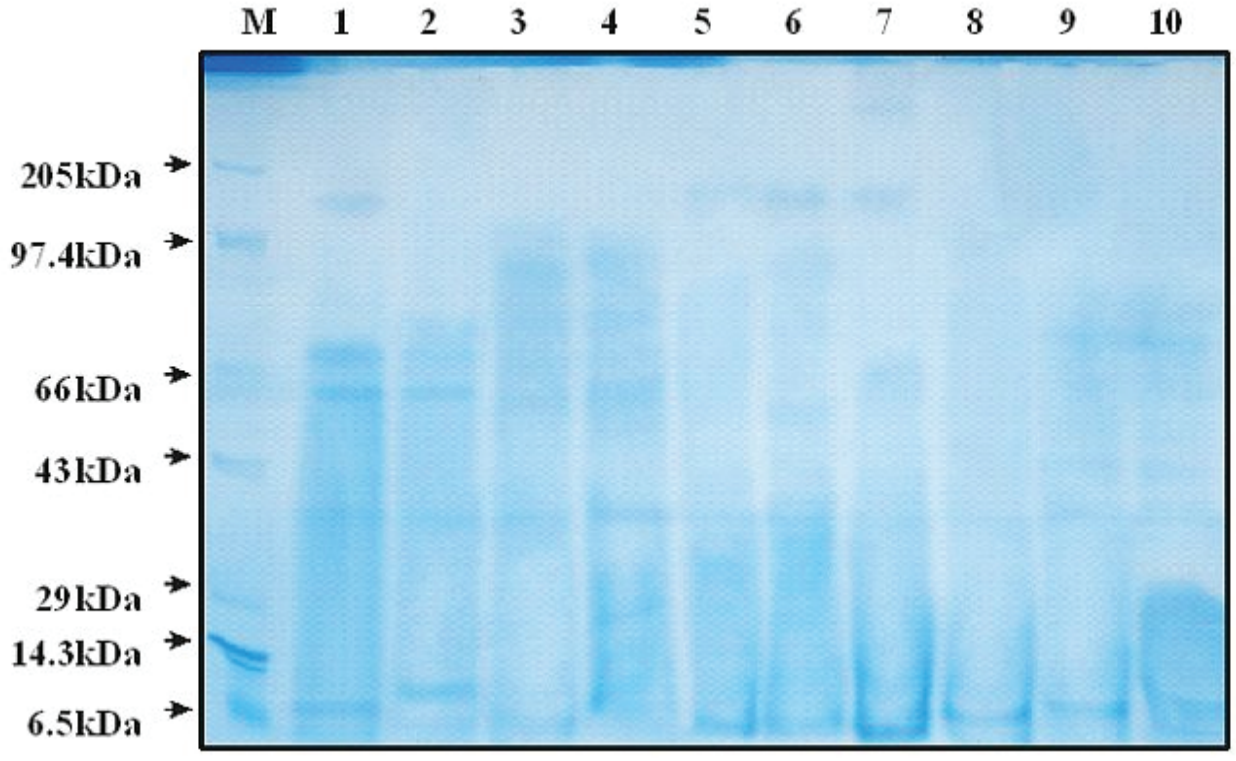

Figure 1. Protein profile in the root of *A. spinosus* under heavy metal stress. M-Marker, Lane 1 - Control, Lanes 2, 3 and 4 - Root treated with lead 100, 150 and 200 µM, respectively, Lanes 5, 6 and 7 - Root treated with cadmium 5, 10 and 15 µM, respectively, Lanes 8, 9 and 10 - Root treated with chromium 100, 150 and 200 µM, respectively

Table 1. Protein profile in the root of A. spinosus under heavy metal stress

	Lane											
Band	1	2	3	4	5	6	7	8	9	10	MW	
	C	Pb1	Pb2	Pb3	Cd1	Cd2	Cd3	Cr1	Cr2	Cr3	(KDa)	Rf
1	+	-	-	-	+	+	+	-	+	-	166.87	0.22
2	-	-	-	-	-	-	-	+	-	-	132.61	0.27
3	-	-	+	+	-	+	-	-	-	-	110.51	0.32
4	-	-	+	+	-	-	-	-	+	+	86.44	0.38
5	+	+	-	+	-	-	+	-	+	+	69.79	0.44
6	+	+	+	+	+	+	-	-	+	+	56.80	0.50
7	-	-	-	+	-	-	-	+	+	+	45. 85	0.55
8	+	+	+	+	+	+	+	+	+	+	31.10	0.66
9	-	-	-	+	+	+	-	-	-	+	22.83	0.74
10	+	+	+	+	+	+	+	+	+	+	11.64	0.92

C- control, Pb1(100 µM), Pb2(150 µM), Pb3(200 µM), Cd1(5 µM), Cd2(10 µM), Cd3(15 µM), Cr1(100 µM), Cr2(150 µM), Cr3(200 µM)

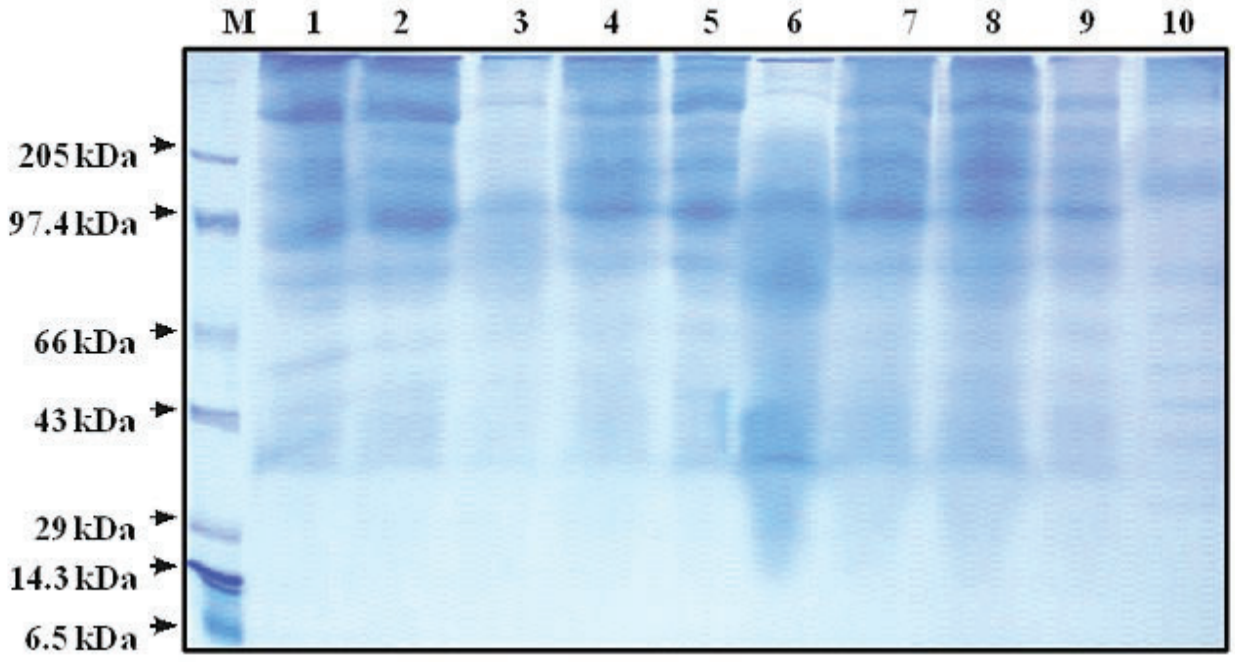

Figure 2. Protein profile in the leaf of *A. spinosus* under heavy metal stress. M-Marker, Lane 1 - Control, Lanes 2, 3 and 4 - Root treated with lead 100, 150 and 200 µM, respectively, Lanes 5, 6 and 7 - Root treated with cadmium 5, 10 and 15 µM, respectively, Lanes 8, 9 and 10 - Root treated with chromium 100, 150 and 200 µM, respectively

Table 2. Protein profile in the leaf of *A. spinosus* under heavy metal stress

Band	Lane										MW	
	1	2	3	4	5	6	7	8	9	10		
	C	Pb1	Pb2	Pb3	Cd1	Cd2	Cd3	Cr1	Cr2	Cr3	(KDa)	Rf
1	-	+	+	+	+	-	-	-	-	-	352.92	0.02
2	+	-	-	-	-	+	+	-	-	-	317.53	0.05
3	-	-	-	-	-	+	-	-	-	-	273.86	0.08
4	+	+	+	+	+	-	+	+	+	-	246.39	0.11
5	-	+	-	+	+	+	+	-	-	-	201.57	0.16
6	-	-	-	-	-	-	-	+	-	-	195.28	0.18
7	+	-	-	-	+	+	+	+	+	-	159.75	0.23
8	+	+	-	+	-	+	-	-	-	-	142.22	0.26
9	-	-	+	+	+	+	+	+	+	-	119.58	0.31
10	+	+	-	-	-	-	-	-	-	-	104.22	0.34
11	-	-	+	+	+	-	+	-	-	+	84.73	0.40
12	+	-	-	-	-	-	-	+	+	-	72.31	0.44
13	+	+	+	+	+	+	+	+	-	+	62.37	0.48
14	-	-	-	+	+	-	-	-	+	+	49.95	0.54
15	+	+	-	+	-	+	+	-	-	-	41.74	0.59
16	-	-	+	-	-	-	-	+	+	+	36.77	0.62
17	+	+	-	+	+	+	+	+	+	-	33.08	0.65
18	+	+	-	+	+	+	+	+	-	-	27.06	0.69
19	-	+	-	-	+	-	+	-	-	-	25.13	0.71
20	+	+	-	+	+	+	+	+	+	-	22.68	0.76
21	-	+	-	-	+	-	-	-	-	-	20.59	0.79

C- control, Pb1(100 μM), Pb2(150 μM), Pb3(200 μM), Cd1(5 μM), Cd2(10 μM), Cd3(15 μM), Cr1(100 μM), Cr2(150 μM), Cr3(200 μM)

control plants recorded five polypeptides having molecular weight 166.87, 69.79, 56.80, 31.10 and 11.64 kDa. The plant treated with varying concentration of Pb^{2+}showed disappearance of two bands at molecular weight of 166.87 and 69.79 kDa and appearance of four polypeptides having molecular weight of 110.51, 86.44, 45.85 and 22.83 kDa. Under different metal treatments of cadmium, it was noticed that only two polypeptides were found to be disappeared (69.79 and 56.80 kDa) where as two new polypeptides became visible with molecular weight 110.51 and 22.83 kDa. Varying metal treatments of chromium caused the disappearance of three bands (166.87, 56.80 and 69.79 kDa) and appearance of four polypeptide bands with molecular weight 132.61, 86.44, 45.85 and 22.83 kDa. Root of *A. spinosus* both treated and untreated plants exhibited a total of ten bands, of which some appeared newly. It was noticed that five new

polypeptides with molecular weight 132.61, 110.51, 86.44, 45.85 and 22.83 kDa appeared in the root of *A. spinosus* after its treatment with varying concentration of Pb^{2+}, Cd^{2+} and Cr^{6+}, respectively.

Two polypeptides with molecular weight 31.10 and 11.64 kDa were found expressed in all the lanes. A new polypeptide with molecular weight 132.61 kDa became visible only under lower concentration of Cr^{6+} whereas another polypeptide band having molecular weight of 166.87 kDa disappeared under all the treatments of Pb^{2+}. The appearance and disappearance of proteins may be due to major reshuffle of protein profile under metal stress which was supported by the findings of Stefanic et al. (2012) and Anju et al. (2014) in tobacco seedlings and papaya seedlings. It was also reported that new bands were formed in treated papaya seedlings because of structural differences in plant seedlings under different concentration of heavy metal toxicity. The disappearance and appearance of some polypeptides in response to heavy metal treatments indicated a direct relationship of polypeptides with heavy metal stress. The newly synthesized proteins have a role in heavy metal tolerance by making biochemical and structural adjustment that enable the plant to cope with heavy metal stress (El-Aref and Hamada, 1998)

The PAGE analysis revealed that the leaf of control plants exhibited the expression of eleven bands with molecular weight ranging between 317.53 and 22.68 kDa. The toxicity of different treatments of Pb^{2+}, suppressed the expression of polypeptide bands with molecular weight 317.53, 159.75 and 72.31 kDa (T1), 317.53, 159.75, 142.22, 104.22, 72.31, 41.74, 33.08, 27.06 and 22.68 kDa (T2) and 317.53, 159.75, 104.22, 72.31 kDa (T3). Eight new bands with 352.92, 201.57, 119.58, 84.73, 49.95, 36.77, 25.13 and 20.59 kDa were found to be appeared after Pb^{2+} treatments. All the Pb^{2+} treatments (T1, T2 and T3) caused complete disappearance of three polypeptide bands having molecular weight of 317.53, 159.75 and 72.31kDa.

After cadmium stress, leaf of *A. spinosus* showed the disappearance of polypeptides with molecular weight 317.53, 142.22, 104.22, 72.31 and 41.74 kDa (T1), 246.39, 104.22, 72.31 kDa (T2) and 142.22, 104.22 and 72.31 kDa (T3). Cadmium stress (T1, T2, and T3) caused complete disappearance of two polypeptides with molecular weight 104.22 and 72.31 kDa and the appearance of eight new bands having molecular weight 352.92, 273.86, 201.57, 119.58, 84.73, 49.95, 25.13 and 20.59 kDa.

A polypeptide having molecular weight 273.86 kDa appeared only under Cd2+ stress (T2) Chromium stress displayed the suppression of peptide bands having molecular weight 317.53, 142.22, 104.22 and 41.74 kDa (T1), 317.53, 142.22, 104.22, 62.37, 41.74 and 27.06 kDa (T2) and 317.53, 246.39, 159.75, 142.22, 104.22, 72.31, 41.74, 33.08, 27.06 and 22.68 kDa (T3). It was observed that four polypeptides present in the leaf of control plants having molecular weight 317.53, 142.22, 104.22 and 41.74 kDa completely suppressed by all the treatments of Cr6+(T1, T2, T3), whereas another polypeptide having molecular weight 195.28 kDa expressed only under Cr^{6+} stress (T1). Heavy metal treated *Amaranthus hybridus* and *Madhuca longifolia* exhibited more number of polypeptides than untreated plants (Farrag et al., 2014). A polypeptide having 98 kDa found suppressed in *Triticum aestivum* under NaCl stress whereas proline was found successful in the protection and upregulation of stress proteins (Ismail, 2014). SDS PAGE was performed in common bean plants

before and after lime treatments to assess variations in the electrophoretic protein banding and summarized that the total number of proteins and band intensity increased in plants after liming (Aldoobie & Beltagi, 2013). Gomes et al. (2012) concluded that proteomic analysis revealed the inhibition of photosynthetic proteins and activation of proteins involved in the defense mechanism to induce tolerance in plants.

The SDS PAGE analysis of *A. spinosus* exhibited visible differences on the appearance and disappearance of polypeptides. When compared the effect of each heavy metal in root and leaf of *A. spinosus*, it was observed that five polypeptides newly appeared in the root but disappearance occurred under Pb^{2+}, $Cd^{2+}$and Cr^{6+} with molecular weight that ranged between 166.87 and 11.64 kDa. The SDS PAGE analysis in leaf of *A. spinosus* showed the appearance of ten bands and disappearance of several bands under heavy metal stress. Several classes of Heat Shock Proteins (HSPs) were identified in plants with molecular weight 110 kDa to low molecular weight HSPs (Vierling, 1991). These HSPs act as molecular chaperones and they contributing to cellular homeostasis under stress conditions and aid in protection, degradation of damaged cell components during abiotic stresses (Joseph et al., 2012). The HSPs are responsible for the protein folding, assembly, translocation and degradation. The heavy metal stress in turn induce conditions similar to water and temperature stress in plants which appear as co stress that activate proteins having molecular weight within the range of HSPs (Knight & Ackerly, 2001) that were found appeared or expressed in the present investigation. The heavy metal stress caused complete disappearance of certain polypeptides in *A. spinosus* and it was noticed that four polypeptides with molecular weight 317.53,166.87 kDa, 159.75 and 72.31kDa were supressed under Pb^{2+} stress, while in Cd^{2+} treatments two bands disappeared with molecular weight 104.22 and 72.31 kDa. The protein profiling under Cr^{6+} treatments exhibited the disappearance of four bands with molecular weight 317.53, 142.22, 104.22 and 41.74 kDa.

The banding pattern of both treated and untreated leaf samples of *A. spinosus* showed a total of 21 bands with molecular size 352.92 to 20.59 kDa. Several polypeptides found to be disappeared in *A. spinosus* under heavy metal stressed condition. Twelve polypeptides (352.92, 201.57, 119.58, 110.51, 86.44, 84.73, 49.95, 45. 85, 36.77, 25.13, 22.83 and 20.59 kDa) were found expressed in *A. spinosus* after the treatment with Pb2+ while the plant samples treated with Cd^{2+} exhibited the appearance of nine bands with 352.92, 273.86, 201.57, 119.58, 84.73, 49.95, 25.13, 21.22 and 20.59 kDa molecular weight. Nine bands appeared under Cr^{6+} treatment with molecular weight 195.28, 132.61, 119.58, 86.44, 84.73, 49.95, 45.85, 36.77 and 22.83 kDa. Plants normally exhibit multiple tolerance mechanisms. It was observed that the seedlings exposed to heavy metal stress acquire the tolerance to lethal and sub lethal temperatures (Prasad, 2004). This may be due to their ability to cope up with co-stress and resultant adaptation at cellular and molecular level. Common induction of several proteins especially HSP was evident in cadmium as well as other abiotic stresses which may be due to the activation of heat shock transcription factors. Czarnecka et al. (1984) opined that some nuclear proteins bind to the promoter region of

a small Hsp causing its expression. HSP are stress proteins normally get induced in response to a wide range of biotic and abiotic stresses in plants. Heavy metal toxicity also favour over expression of plasma membrane transporters involved in the accumulation of ions.

In general, sixteen bands appeared in *A. spinosus* with molecular weight 352.92, 273.86, 201.57, 195.28, 132.61, 119.58, 110.51, 86.44, 84.73, 49.95, 45.85, 36.77, 25.13, 22.83, 21.22 and 20.59 kDa. SDS PAGE analysis exhibited more number of polypeptides in leaf than root. These findings were in accordance with the findings of Rout & Sahoo (2012) that leaf of *Withania somnifera* treated with heavy metals showed the presence of several new protein bands with molecular weight ranging from 98.14 kDa to 17.49 kDa and some polypeptide bands even showed more expression. Two new polypeptides (147.82 and 131.55 kDa) appeared in root where as one polypeptide with 26.88 kDa disappeared during iron treatment. Jomova & Morovic (2009) concluded that the increased proteins have a major role in cell defence reactions.

Conclusions

SDS-PAGE analysis exhibited visible changes in electrophoretic protein bands and the banding pattern revealed the appearance of new bands and disappearance of existing bands in metal treated plants as compared to control. SDS page revealed induction of new polypeptides in both plant parts. The metal homeostasis is a complex mechanism which involves an appraisal of sensing stimuli, cell response and signalling followed by effective defense strategies. The modulation of histological, physiological, biochemical and molecular status of the organism impart better tolerance to toxic heavy metals. A multiple approach to the identification and detection techniques of environmentally pertinent metals coupled with metallomics and metalloproteomics is a better strategy for elucidating a fruitful conclusion of heavy metal tolerance in plants. Moreover, environmental biotechnology like phytoremediation, phytomining and biofortification is eco-friendly and cost effective future perspective of metal tolerance in flora which in turn can tackle the problem of heavy metal pollution prevalent in the biosphere to some extent.

Conflict of interest: Author declares no conflict of interest.

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