

ISSN: 2220-4822

# Lead induced changes in biomarkers and proteome map of Chicory (*Cichorium intybus* L.)

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## ABSTRACT

Lead (Pb) toxicity is a serious environmental problem as it affects the food production by interfering plant growth and development, thus declines the production yield. In the present research work, *Cichorium intybus* L. plants were subjected to different concentrations of Pb (0, 100, 200 and 300 $\mu$ M) upto 46days to determine the oxidative stress. The length of root and shoot, accumulation of biomass were estimated along with the changes in biomarkers (H<sub>2</sub>O<sub>2</sub> and TBARS). Further proteomic analysis of chicory leaves (46days old) at 300 $\mu$ M Pb concentration was done to identify the proteins of interest. The root growth increased significantly in a concentration-dependent manner however; shoot growth, biomass accumulation declined significantly with Pb stress compared to control. Changes in biomarkers (H<sub>2</sub>O<sub>2</sub> and TBARS) content elevated with the increment in the concentration of metal treatment but exhibited a gradual decline at 300 $\mu$ M Pb treatment. Proteomics data of 46days old chicory plants under 300  $\mu$ M Pb stress analyzed by PDQuest software detected approximately 168 protein spots on each gel and 81 spots were differentially expressed in which 16 were up-regulated and 13 were down-regulated. The present study suggested that chicory possess a strong antioxidative defense system to combat Pb stress and thus could be explored for cultivation in Pb contaminated soils.

**Received:** April 16, 2020  
**Revised:** May 22, 2020  
**Accepted:** May 27, 2020  
**Published:** June 13, 2020

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**KEYWORDS:** Biomarkers, Chicory, Lead, Oxidative stress, Proteomics

## INTRODUCTION

With the increasing population and environmental pollution, agricultural sector is affected at a very faster pace due to deterioration of soil quality and other edaphic factors by excessive use of fertilizers, pesticides and seed disinfectants which ultimately declines the crop yield. Besides these mal-agricultural practices, rapid industrialization and urbanization are also significant factors contributing to widespread pollution that are hazardous to environment. All these mal-practices leads to bioaccumulation and biomagnification of toxic metals in the in the soil and water bodies that has become nuisance to our sustainable development [1]. Lead (Pb) is a substantial heavy metal distributed largely in the environment and poses an extensive damage to the humans by causing nephropathy, anaemia and central nervous system disorders [2-4]. Pb is introduced in the soil throughsmelting, gasoline, storage battery, explosives, paints, synthesis of tetraethyl-lead and plating, mining, ceramic sewage sludge, ammunition, fertilizers and glass industries [5&6]. Pb toxicity affects plant growth and development by altering various physiological and biochemical pathways [7,8&9]. It leads to oxidative stress by production

of reactive oxygen species (ROS) which causes disruption of membrane and ultimately cell death [10,11&12]. Moreover, Pb toxicity also interfere osmolytes and antioxidative defense system by altering their expression levels [13,14,15&16]. In order to combat these abiotic stresses, plants have the ability to stimulate various defense mechanisms by activating expression levels of osmoprotectants, antioxidant enzymes as well as modification at the proteome level. Proteomics is an advanced technique nowadays used in the analysis of proteins crucial for the normal functioning of the cell. It is a robust technique to not only unravel the novel stress proteins in plants but also provides a complete proteome map at all levels of organization under stress conditions [17].

In this perspective, the need of the hour is to focus on such plants that can resist the harsh climatic conditions and *Cichorium intybus* L. (Chicory) is one of the important plants that have achieved a rapid momentum in the nutraceutical sector because of its high food and medicinal value. However, there is currently no data available on the proteomic profiling of chicory under heavy metal stress, therefore, it is in this backdrop that the current investigation was undertaken to unravel the

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modifications in biomarkers and at proteome level in chicory under Pb stress.

## MATERIAL AND METHODS

### Plant Growth and Treatment

Seed material of chicory (*Cichorium intybus* L.) was obtained from Hamdard University, New Delhi, India. Surface sterilization of viable seeds for 15 min, was done using sodium hypochlorite (5% v/v) followed by rinsing with distilled water and later seeds were soaked in deionized water for 1h. Soaked seeds were sown in 0.5kg acid washed autoclaved sand in plastic pots of 8cm diameter. Liquid fertilizer (Hoagland's solution; pH 6.5) was utilized throughout the experiment for the growth of seedlings [18]. Later, pots were kept under controlled conditions (photon flux intensity  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; relative humidity 60-70%; temp.  $22.5 \pm 1.5^\circ\text{C}$  at daytime;  $18.5 \pm 1.5^\circ\text{C}$  at night time). For treatment, solution of lead nitrate ( $\text{PbNO}_3$ ) prepared in Hoagland's solution were given at various concentrations (0, 100, 200 and  $300 \mu\text{M}$  for 23 days after sowing (DAS). Experiment was conducted in replicates and was arranged in a completely randomized block design and sampling was done at 46 days after sowing (DAS).

### Growth Analysis

The growth of chicory seedlings (10 seedlings/ replicate) were assessed by measuring root, shoot length and biomass accumulation and were expressed in mg/seedling.

### Quantification of Biomarkers

#### *Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and Thiobarbituric acid reactive substances (TBARS) estimation*

The accumulation of  $\text{H}_2\text{O}_2$  in leaves was estimated following the protocol of Velikova et al. [19] with slight modifications. Leaves were homogenized in liquid nitrogen by adding 5ml of TCA [0.1% (w/v)]. At room temperature, the homogenate was centrifuged for 15 min at  $12,000 \times g$ . After centrifugation, 0.5 ml of 1M potassium phosphate buffer (pH7.0) and 1 ml of KI (1M) were added to the supernatant and the absorbance was taken at 390 nm. Lipid peroxidation usually measured in terms of thiobarbituric acid reactive substances (TBARS) was determined according to the protocol of Heath and Packer [20].

The quantity of  $\text{H}_2\text{O}_2$  and TBARS content was estimated using the following formula

$$\text{H}_2\text{O}_2 (\mu\text{mol/g FW}) = 1 + 227.8 \times \text{O.D.}_{390}$$

$$\text{TBARS content (nmol/g FW)} = (\text{A}_{532} - \text{A}_{600}) \times \frac{\text{V} \times 1000}{\epsilon \times \text{W}}$$

“V” denotes extraction volume; “W” denotes weight of fresh tissue

“ $\epsilon$ ” denotes molar extinction coefficient for MDA ( $\epsilon=155 \text{ mM}^{-1}\text{cm}^{-1}$ )

### Proteomic Profiling

#### *Protein extraction and quantification*

Protein extraction was done following the protocol of Bagheri et al. [21]. Fresh leaf material was mashed to a fine powder using liquid nitrogen in pre-chilled pestle and mortar and homogenized in a solution (40 mM (w/v) Tris-HCl, pH 7.5, 2 mM (w/v) EDTA, 0.07 % (v/v)  $\beta$ -mercaptoethanol, 2% (w/v) PVP, 1% (v/v) TritonX100 and 1% (w/v) PMSF). Later, the homogenate was centrifuged for 60 min at 20,000 rpm ( $4^\circ\text{C}$ ). Supernatant obtained was blended in the ratio of 1:2 with 10% (w/v) TCA and 0.07% (w/v)  $\beta$ -mercaptoethanol, prepared in acetone, and left at  $-20^\circ\text{C}$  overnight. Next day, the mixture was again centrifuged for 15 min at 15,000 rpm. The obtained pellet was then washed with chilled acetone containing 0.07 % (v/v)  $\beta$ -mercaptoethanol and 2 mM EDTA. Incubation of pellet was done using chilled acetone [100% (v/v)] for 5 h and then was dried under vacuum. Quantification of protein was done using Bradford reagent [22] and Bovine Serum Albumin (BSA) as a standard. Later,  $150 \mu\text{g}$  of protein was loaded on IPG strip (11cm; pH=4-7 NL) for 55,000Vh. Equilibration of IPG strip was done in a solution containing 130mM DTT and 135mM iodoacetamide. 2<sup>nd</sup> dimensional run was carried out using 12% SDS-PAGE at 100volts using previously obtained IPG strip. Staining of gel containing proteins was done using Coomassie Blue G-250 and later destained in distilled water. Estimation of spot density was performed using PDQuest software version 8.0, (Bio-Rad, USA). Spots intensity were measured on the basis of their relative volume, that was estimated by the ratio of the volume of a single spot to the whole set of spots under stress. Spots with significant change more than two-fold in abundance and with reproducible occurrence in replicates were used for further study.

### Statistical Analysis

Experiments were conducted in triplicates and the data were presented as average  $\pm$  standard deviation (SD). Data were analysed by GraphPad Prism 7.0 software for one-way ANOVA. Mean differences were estimated at 5% probability level using Tukey's post hoc test.

## RESULT

### Analysis of Growth Parameters

The effect of different concentrations of Pb treatment on plant growth and biomass accumulation of chicory seedlings at 46 days are presented in Table 1. Upon the treatment of Pb, changes in root growth were significant with respect to control as it increased initially at  $100 \mu\text{M}$  to  $2.27 \pm 0.002$  but decreased non-significantly at  $200 \mu\text{M}$  to  $2.22 \pm 0.001$  but thereafter showed a significant increase of  $2.89 \pm 0.001$  at  $300 \mu\text{M}$  respectively. The shoot growth initially showed decrease with

each increment of Pb treatment and the maximum decrease was observed  $4.25 \pm 0.001$  at  $100 \mu\text{M}$  with respect to control. Biomass accumulation initially showed a non-significant increase with increase in concentration of Pb treatment but thereafter showed a significant decrease of  $0.0009 \pm 0.002$  at  $300 \mu\text{M}$  respectively with respect to control.

## Biomarker Analysis

### Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and TBARS content

When subjected to different concentration of Pb treatments, the  $\text{H}_2\text{O}_2$  level enhanced and the significant increment was observed at  $200 \mu\text{M}$  Pb stress ( $110.12 \pm 5.1 \mu\text{molg}^{-1}\text{FW}$ ) compared to control (Figure 1a). Similarly, the present data showed that the TBARS content increased with increment in Pb treatment and the maximum value was found to be  $3.12 \pm 0.08 \text{nmolg}^{-1}\text{FW}$  at  $100 \mu\text{M}$  compared to control (Figure 1b).

## Proteomic Profiling

In the present study, the leaf materials (control and treated samples) were collected after 46 days for proteomic analysis. Protein separation was done in the first dimension using immobilized pH gradient gels with a pH range of 4-7,  $200 \mu\text{g}$  of protein was located on an 11cm IPG strip (NL) followed by second dimension protein separation using 12% SDS-PAGE.

**Table 1: Effect of different concentrations of Pb treatments (0, 100, 200 and  $300 \mu\text{M}$ ) on root, shoot length, biomass accumulation of 46-day old *Cichorium intybus* plants**

Concentration ( $\mu\text{M}$ )	Mean Root Length (cm/seedling)	Mean shoot Length (cm/seedling)	Biomass accumulation (BA) (mg/seedling)
0	$2.19 \pm 0.1$	$4.8 \pm 0.002$	$0.001 \pm 0.0002$
100	$2.27 \pm 0.002^{**}$	$4.25 \pm 0.003^*$	$0.0013 \pm 0.0002^{ns}$
200	$2.22 \pm 0.001^{ns}$	$4.72 \pm 0.02^{ns}$	$0.0011 \pm 0.0001^{ns}$
300	$2.89 \pm 0.001^{***}$	$4.41 \pm 0.001^*$	$0.0009 \pm 0.0002^{***}$

Data represent the mean  $\pm$  SE of three different experiments (n=10). ns- non-significant; \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001 and \*\*\*\*P < 0.0001

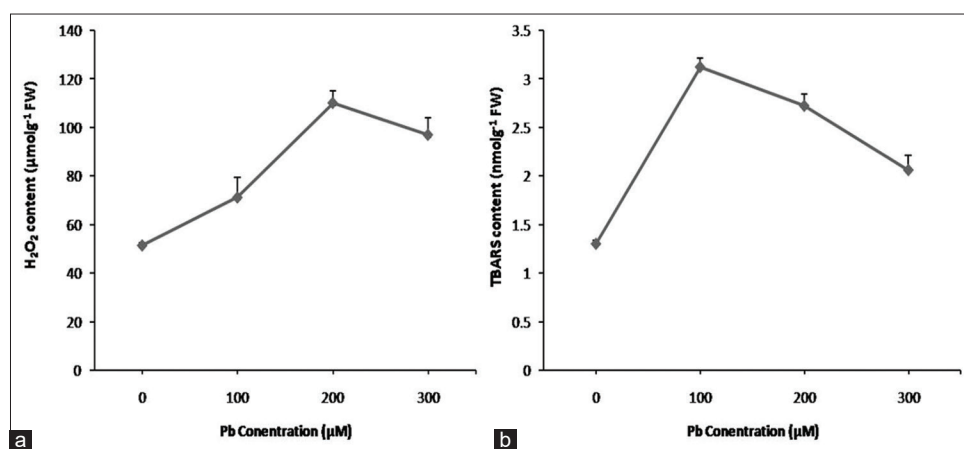
Visualization of separated proteins was done by colloidal Coomassie Brilliant Blue (CBB) stain and the patterns of spots were analyzed using image analysis software (PDQuest™, Bio-Rad, USA). Proteomics data of 46 days old chicory plants under  $300 \mu\text{M}$  Pb stress analyzed by PDQuest™ software detected approximately 168 protein spots on each gel (Figure 2). 81 spots were differentially expressed under Pb treatment in which 16 were up-regulated and 13 were down-regulated. The intensity of spots which exhibited statistically significant change (2-fold) was represented in Figure 3. Scatter plot analysis was performed which showed 2-fold up-regulation or down-regulation of proteins (Figure 4). Present data also showed that approximately 25 distinct spots were considered of high interest due to major alterations in terms of their intensities on gels. Summary of protein IDs with SSP number, molecular weight (MW), PI and fold change with respect to control of chicory seedlings grown under Pb heavy metal stress is depicted in Table 2.

## DISCUSSION

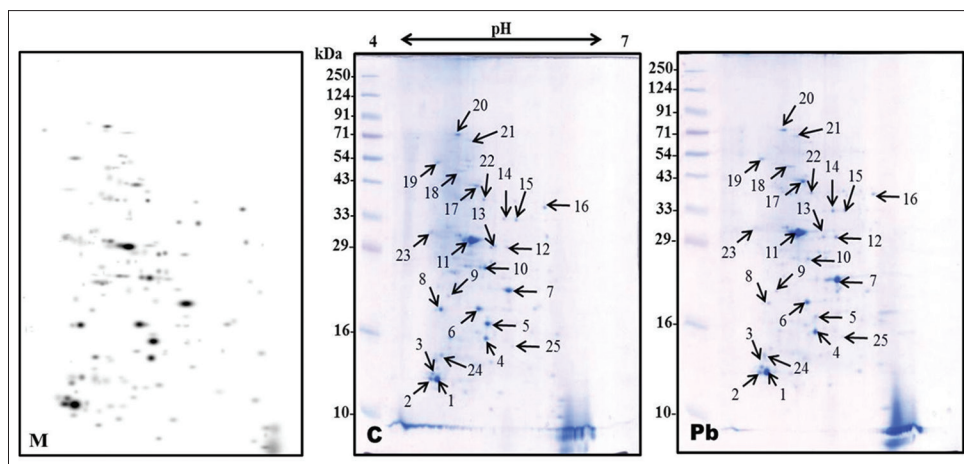
### Analysis of Growth Parameters

Analysis of plant growth parameters subjected to metal stress forms the basis of plant stress physiology and is considered as an important index in phytoremediation process.

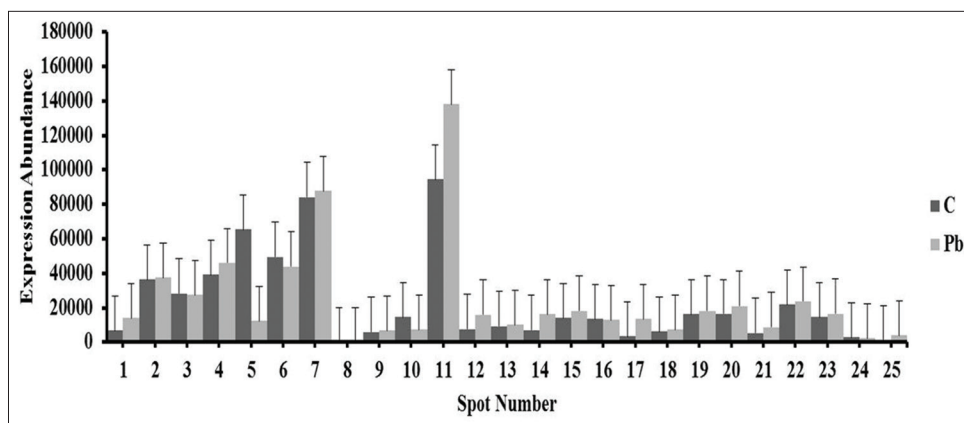
The present data revealed that Pb stress affects the growth of chicory plant but the inhibition was more significant in shoots compared to root. The inhibition of shoot growth with increasing Pb concentration might be due to the more Pb-uptake in shoots that causes hindrances in various physiological pathways and ultimately suppresses the growth and development of the plant [23]. In the current study, the root growth of chicory plants was significantly increased in response to Pb heavy metal exposure which was evidenced by significant decrease in shoot length with the increasing concentration of Pb as compared to control plants. Pb inhibited the shoot growth more than root growth. Similar results of growth inhibition with increase in concentration were also observed in *Triticum sativum* [24] and *Elsholtzia argyi* [25] under Pb stress. Many studies have reported



**Figure 1: Effect of Pb concentrations (0, 100, 200 and  $300 \mu\text{M}$ ) on (a) hydrogen peroxide content (b) and TBARS content of *Cichorium intybus* at 46 days after sowing: DAS**



**Figure 2:** 2-DE images representing differentially expressed proteins in control (c) versus Pb stress at 46 days after sowing in the leaves of *Cichorium intybus*



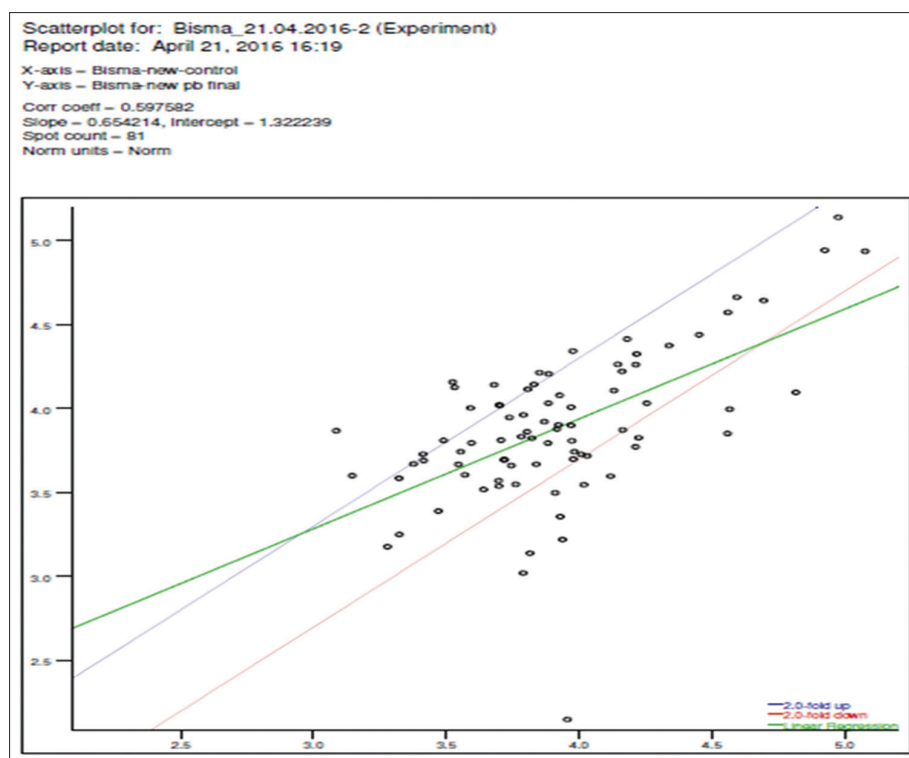
**Figure 3:** Plot of the 2DGE protein spots intensities indicating expression levels, for proteins extracted from chicory leaves after exposure to Pb (300µM) stress conditions

that higher levels of metals restrain aquaporins in higher plants, altering the membrane permeability that ultimately affects their growth [26&27]. Moreover, plant growth inhibition by Pb stress at higher concentration might be due to inhibition of mitotic index as was previously observed with Cd and Pb stress [28]. Further, biomass estimation is regarded as important indices to determine the performance of plants exposed to heavy metal stress. In the present study, Pb treatments reduced chicory plant growth rate and thus decline the overall biomass production. Previous observations of decreased plant biomass under Pb treatment were previously noticed in *Lathyrus sativus* L. [29], *Fagopyrum kashmirianum* and in *Vicia faba*, *Pisum sativum* and *Phaseolus vulgaris* [30]. Reduction in chicory plant biomass at higher Pb levels may be correlated to high accumulation by plants and the cells might need more energy to overcome with the high concentration of metals [31&32].

**Biomarker Analysis**

Lipid peroxidation caused internal damage to the membranes due to abiotic stresses particularly by heavy metal stress [33]. Our study revealed that H<sub>2</sub>O<sub>2</sub> production increased at each level of Pb stress initially upto 200µM but decreased at

higher concentration (300µM) compared to control plants suggesting a regulatory role of H<sub>2</sub>O<sub>2</sub> in the cross talk of stress signaling pathway and redox metabolic signals. The present data revealed that lipid peroxidation in leaves of chicory plants measured as MDA content enhanced with increasing Pb concentrations initially and then declined at 300µM compared to the control and the reason might be to the enhanced activities of antioxidant enzymes and therefore, the cell membrane damage could be minimized. MDA is a by-product of lipid peroxidation caused due to disruption of membrane by oxidative stress and is considered as an important biomarker in stress biology [34&35]. The ROS generated during oxidative stress causes removal of hydrogen ions from unsaturated fatty acids resulting in the production of lipid radicals and reactive aldehydes [36]. Present data suggests that membrane damage has occurred in chicory plants due to Pb treatment and besides the enhanced level of lipid peroxidation might be due increased activity of antioxidative enzymes to scavenge H<sub>2</sub>O<sub>2</sub> radicals and thus minimizes the cellular damage [37]. Enhanced lipid peroxidation has also been reported in *Cycopersium esculentum* [32], *Brassica* [38], *Vigna radiate* [39] and in *Fagopyrum species*.



**Figure 4:** Scatter plot showing differential expression patterns of proteins in chicory under Pb stress as compared to control

**Table 2:** Summary of protein IDs with SSP number, molecular weight (MW), PI and fold change with respect to control of *Cichorium intybus* seedlings under Pb stress

S. No.	Spot ID	SSP	MW	PI	C	Pb
1.	1	2101	12.10	4.71	✓	1.06
2.	2	0103	12.04	4.33	✓	0.031
3.	3	0104	12.51	4.37	✓	0.027
4.	4	6201	16.14	5.28	✓	0.174
5.	5	6303	17.94	5.29	✓	0.809
6.	6	5301	20.00	5.20	✓	0.747
7.	7	8401	24.00	5.50	✓	0.042
8.	8	1302	20.45	4.6	✓	×
9.	9	2401	22.7	4.76	✓	0.122
10.	10	6501	27.01	5.22	✓	0.488
11.	11	5602	31.00	5.1	✓	0.459
12.	12	7603	29.92	5.49	✓	1.10
13.	13	7602	30.12	5.36	✓	0.094
14.	14	7701	34.21	5.45	✓	1.31
15.	15	8701	34.05	5.62	✓	0.310
16.	16	8802	36.00	6.10	✓	0.053
17.	17	5802	50.44	5.08	✓	2.94
18.	18	3701	31.77	4.87	✓	0.141
19.	19	1901	70.00	4.6	✓	0.120
20.	20	3902	134.11	4.90	✓	0.285
21.	21	5902	117.66	5.09	✓	0.20
22.	22	5804	49.63	5.17	✓	0.08
23.	23	0702	32.18	4.35	✓	0.0147
24.	24	1103	13.02	4.61	✓	0.170
25.	25	8303	18.07	5.64	1400.1	1.85

### Proteomic Profiling

In the present study, proteomics profiling was done to unravel the proteins involved in Pb tolerance in chicory. Here, we report the first proteomic studies on chicory grown in Kashmir Himalaya to

understand its tolerance mechanism against Pb stress. The present study revealed that Pb induces oxidative stress in the leaf of chicory. Pb stress revealed the differential expression of ~30 proteins, and most among them were up-regulated under Pb stress. Up-regulated proportion of proteins was 64% whereas down-regulated proportions were 52% of the total proteome under Pb stress. Besides, it is known fact that proteins associated with plant stress responses and adaptations to metal toxicity is always accompanied with modifications in proteomic changes. Thus, proteomics provides us a better understanding to elucidate the possible relationships between proteins abundance and plant stress adaptation. Proteomics helps in elucidating the proteins that are either up- or down-regulated with respect to various stresses and proves to be a useful technique in evaluating the early events that take place during heavy metal stress.

### CONCLUSION

In conclusion, the present data suggests that Pb stress interfere morpho-physiology of chicory as evident from growth inhibition, decreased biomass accumulation, lipid peroxidation and increase accumulation of hydrogen peroxide. Besides, chicory modulated its proteome profile very efficiently under Pb stress by down-regulating and up-regulating the expression of several proteins. Such proteins are having roles in various cell functions including imparting of defence against Pb stress thus could be used to detoxify Pb contaminated soils.

### ACKNOWLEDGMENT

The authors are highly acknowledged to University Centre for Research and Development (UCRD), Chandigarh University, Mohali, Punjab, India for the support.



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