

Proteomic alterations in various plant tissues of maize under induced chromium stress

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Abstract

Heavy metal contamination is becoming a major cause of pollution in the environment, impacting humans, animals, and plants directly. Because of its widespread use in the tanning industry, chromium (Cr) regarded as a highly dangerous environmental toxin. The goal of this study was to investigate growth and proteins changes in different plant tissues (leaves, shoots, and seeds) of two maize cultivars (NMH-360 and DKC 61-42) under the stress of Cr (0, 50, and 150 ppm Cr). Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was used to observe plant proteome modification in response to Cr stress. Results revealed that at 150 ppm, both maize cultivars showed a decrease in plant growth attributes. Furthermore, it was noticed that the plant proteome changed in response to Cr stress in leaf and shoot tissues. A few proteins were up-regulated (70 kDa in C₁ variety), while others were down-regulated (154, 140, 115, 80, 70 and 53 kDa in C₁ cultivar shoots while 154, 65, 60, 17 kDa bands in shoots of C₂ cultivar). Some protein bands were induced at 50 ppm, some at 150 ppm, and yet others at both concentrations. The findings of this study could aid in the selection of Cr-tolerant plant cultivars as well as the design of new protein biomarkers that can be utilized as a monitoring tool in heavy metal stress responses.

Keywords: chromium; gel electrophoresis; growth; maize; protein bands

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Introduction

Heavy metal stress, one of the principal abiotic stresses, has increased environmental contamination in recent years (Aslam *et al.*, 2023). Industrial wastewater, phosphate fertilizers, and agrochemicals are the primary sources of these metals in soil, air, and water (Yasin *et al.*, 2023). Heavy metal stress symptoms are comparable to those of critical nutrient deficit, including reduced biomass, poorer growth, necrotic patches on leaves, and root damage (Kieffer *et al.*, 2009). In recent years, the impact of heavy metal on living creatures has piqued people's interest (Raju *et al.*, 2013; Prajapati and Meravi, 2014; Sayadi and Rezaei, 2014).

Phytoremediation is an approach for employing plants to alleviate contaminated soils, water, and sediments from various types of contaminants. It is a low-cost and environmentally beneficial technique (Ferreiro *et al.*, 2014). The use of phytoremediation is based on hyperaccumulator plants' phytoextraction process. Metals are accumulated in the above-ground harvestable sections of these plants (Nakbanpote *et al.*, 2010). Metal accumulation is used by hyperaccumulators in nature as a defence against diseases and herbivores. As a result, they have evolved a distinct mechanism from non-hyperaccumulator plants (Leitenmaier and Kupper, 2013).

Pakistan is a rapidly growing agricultural country with the world's largest canal irrigation system (Mahmood *et al.*, 2007). Pakistan, like other developing countries, has faced serious environmental challenges in the last decade as a result of heavy metal poisoning. The growing number of tanneries in Pakistan is one of the main sources of heavy metal effluents (Waseem *et al.*, 2014). Leather processing tanneries in underdeveloped nations discharge their effluents untreated, allowing pollutants in dissolved form to permeate the surrounding soil (Zulfiqar *et al.*, 2011). In comparison to Cr (III), Cr (VI) is more hazardous and mobile, and it is primarily produced by industries. In tomato, canola, and watermelon, chromium causes a reduction in plant height, number of leaves, root length, fresh and dry weight of root and shoot (Akinci and Akinci, 2010; Shekar *et al.*, 2011). Because of these characteristics, the scientific community considers Cr (VI) to be a priority contaminant (Desai *et al.*, 2008).

After wheat and rice, maize (*Zea mays* L.) is the world's third most significant cereal crop in terms of yield (Iqbal *et al.*, 2014). Maize covers 0.94 million hectares in Pakistan, with a total grain yield of 1.56 million tonnes (Humbert *et al.*, 2013). Because of its year-round output, it is known as the "Queen of Cereal Crops" (Singh *et al.*, 2012). Maize variants have been reported to be more tolerant of tropical and subtropical climates. It can be used as a model to investigate the effects of various stresses (Hussain *et al.*, 2013). Maize genetic diversity has a significant function in maize breeding (Qi-Lun *et al.*, 2008). Knowing the amount and distribution of genetic variation within and among maize varieties will help anticipate the degree of inheritance, or variation, that is necessary for maize breeding (Duan *et al.*, 2006).

To cope with environmental challenges, plants modulate their proteomes. It is critical to have a thorough understanding of plant protein composition, as it plays a critical role in overcoming environmental problems such as heavy metal stress. Proteins are big molecules that are subjected to posttranslational alterations that cannot be assessed through genetic research (Komatsu *et al.*, 2014). Proteomic investigations are useful for identifying the presence of specific proteins and determining their function in various environments (Cvjetko *et al.*, 2014).

Proteins are widely separated and analysed using one-dimensional and two-dimensional approaches (Cvjetko *et al.*, 2014). Electrophoresis of sodium dodecyl sulphate polyacrylamide gels has been useful in determining differences in chemical and physical properties of proteins. This method has been shown to be one of the most relevant metrics for studying total and storage proteins in plants. Several methods have been used in the past, but the quality of the results is dependent on the isolation of rare bands and the amount of protein extracted using SDS-PAGE (Ranjan *et al.*, 2012).

The purpose of this study is to investigate the protein profiles of maize cultivars with varying protein expression patterns in response to various levels of induced chromium stress. This study's findings will contribute to a better understanding of the mechanisms involved in metal toxicity, particularly Cr toxicity. It may help in the identification of new protein biomarkers that may be used to monitor heavy metal stress responses.

Materials and Methods

Plant material and experimental design

Seeds of two commercial maize cultivars (NMH-360 and DKC 61-42 named as C₁ and C₂ respectively) were collected from Federal Seed Certification and Registration Department, 4-Lytton Road Lahore. The experiment was setup in wire house of Department of Botany, University of the Punjab, Lahore. Seeds were sown in triplicates in pots filled with fertile soil taken from Botanical Garden of University of the Punjab. The temperature of the wire house was 28-30 °C and plants got proper sunlight in the wire house. Plants were irrigated on regular basis. Gentle digging of soil was done periodically for the proper aeration of soil. Germination of seeds started after one day. Emergent seedlings were transplanted as three replicates per treatment to the Cr treated soil pots after one week.

Transplantation in chromium treated soil

A weighed amount of air-dried sieved homogenous garden soil (1 kg) was taken and transferred into plastic pots of 6-inch diameter. Chromium was supplied through soil in different concentrations (0, 50, 150 ppm). Chemical grade K₂CrO₄ was used as Cr (VI) source. Three replicates per treatment were used in this study.

Plant sampling for growth parameters.

Harvesting of plants was done after 25 days of growth and growth parameters were measured by the end of the experiment. Plants were cleaned with distilled water to remove soil residues. Growth parameters such as root length, shoot length, fresh and dry weight of root and shoot and total number of leaves per plant were recorded.

Growth attributes

Root and shoot length of each plant was measured in centimetres (cm) from base of the to the tip longest root and shoot by using a meter rod and then the mean values of triplicates were calculated.

Number of leaves

Number of leaves of triplicates was counted and recorded.

Fresh and dry weight

Fresh weight of root and shoot of 25 days old replicate of each plant were measured in grams. Dry weight was taken after drying root and shoot of each plant replicate for 72 h in a 70 °C incubator (Ahsan *et al.*, 2007).

Extraction of protein

Plants from each treatment were harvested after 25 days' period of growth. Plants were washed with de-ionized water. Washing process of fresh leaves, stems and roots was done immediately to avoid protein degradation. Plant samples were stored at -80 °C for further use.

Extraction of protein from seeds

Seeds of both varieties were grown for three days in Petri dishes soaked with distilled water in control and treated with 50 and 150 ppm concentrations of chromium in experimental. Three replicates per treatment were prepared. These Petri dishes were then incubated in dark at 25 °C for 72 h (Gianazza *et al.*, 2007). For the extraction of seed proteins, whole seed was crushed and ground to fine powder with pestle and mortar. Around 0.1-gram seed powder was put into 1.5 ml micro-tube. For the extraction of proteins from powder, 50 mM phosphate extraction buffer (700 µl) of pH 7.0 was added to powder as an extraction liquid. The slurry was then vortexed for 5 minutes and centrifuged at 14,000 rpm in Eppendorf centrifuge 5417R for 10 minutes (Sardar *et al.*, 2022). Supernatant was stored at -20 °C for further use in electrophoresis. The protein concentrations were estimated by Bradford method. All the chemicals were mixed and the final volume was made up to 800 ml, the pH was adjusted to 7.0 and later the final volume of solution was made up to 1 liter.

Extraction of protein from leaves and stem

The protein extraction procedure was reported by Verbi *et al.* (2005). Plant sample (leaves stem) were ground in liquid nitrogen using a pre-chilled pestle and mortar. The proteins were extracted by adding 1.0 g of plant tissue to 3 ml extraction solution (1 mol/L Tris-HCl (pH 6.8), 10% (w/v) SDS, conc. glycerol, B-mercaptoethanol, and high-purity water). The sample was mixed thoroughly by vortexing for 20 minutes, and the remaining insoluble material was removed by centrifugation at 97500 rpm in Eppendorf centrifuge 5417R for 5 minutes at 4 °C. these samples were stored at -80 °C until used.

Acetone preparation

The Trichloroacetic acid acetone precipitation method was adopted in the sample preparation. Briefly, a measured volume of one ml of plant tissue lysate was added in 8 ml ice cold acetone with 1 ml TCA added. Precipitation was done at -20 °C for an hour. Centrifugation was done at 4500 rpm for 40 minutes at 4 °C, supernatant was discarded. Pellet was washed with 1 ml ice cold acetone. Centrifugation was done for 40 min at 4 °C. Supernatant was discarded and pellet was dried at room temperature. Pellet was dissolved in rehydration buffer by pipetting up and down to break up pellet. The sample was kept at 4 °C for 1 hour and vortexed after every 10 minutes. It was centrifuged at 40,000 rpm for 10 minutes at room temperature. The resultant supernatant was stored at -20 °C until it was used for electrophoresis. The protein concentrations were estimated by Bradford method (Zhu *et al.*, 2009). All the chemicals were dissolved in deionized water and final volume of the solution was made up to 5 ml.

Protein quantification

Total protein content in tissue lysate was estimated by Bradford assay using Bovine Serum Albumin as standard (Bradford, 1976). 100 ml of 85% H₃PO₄ was added in working reagent containing 100 mg coomassie blue G250 in 50 ml ethanol, then diluted to 1000 ml with distilled H₂O. A standard graph was plotted against known concentration of BSA ranging from 10 µg to 200 µg on x axis while absorbance (595 nm) was taken on y axis accordingly. The protein samples were serially diluted within the range of standard curve to calculate the concentration of protein in test samples.

Preparation of electrophoretic gel

Gel electrophoresis of proteins provides information about the molecular size, amount, and purity of a protein sample (Zada *et al.*, 2013). It was carried out in Mini PROTEAN® Tetra Cell system from Bio-Rad using a modification of the procedure (Laemmli, 1970). Therefore, 10 µg of the extracted proteins, quantified by Bradford method, were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel. Two vertical glass plates for gel were fixed together. The resolving gel consisted of 1% by weight N,N-methylene-acrylamide in 1.5 M Tris-HCl buffer (pH 8.8) with 10% SDS and 29% acrylamide. The gel was polymerized by adding 4 µl

tetramethylethylene-diamine (TEMED) and 100 μ l ammonium per sulphate (APS). The stacking gel comprised of 1% N.N-methylene-acrylamide in 0.5M Tris-HCl buffer (pH 6.8) with 10% SDS. The gel was polymerized by pouring 3-6 μ l tetramethylethylene-diamine (TEMED) and 30 μ l ammonium per sulphate (APS).

Preparations of different solutions for SDS-PAGE electrophoresis

The gels were run in tris-glycine buffer (the 1x working solution of tris-glycine buffer contain 25 mM Tris-Cl, 250 mM glycine and 0.1% SDS) at 60V until sample entered resolving gel, the voltage was increased to 120V until the dye reached at the bottom of the gel. Later the gels were put in fixative (30% ethanol, 10% acetic acid) over-night and protein bands were visualized by staining with colloidal coomassie blue stain (100 g ammonium sulphate, 1.2 g coomassie blue R250, 100 ml 85% phosphoric acid in H₂O to final volume 800 ml, methanol was added upon use in 1:4v/v i.e., methanol: dye, Thermo Scientific, Illinois) and the gels were destained (5% methanol and 7% acetic acid).

Gel image analysis

ImageQuant TL (GE Healthcare) version 1.1 was used to analysed all 1-D gels. This software is an easy tool for researchers to analysed their 1-D gels. Lanes and bands were defined. Bands were quantified according to molecular weight assigned to markers. Automated analysis eliminate subjectivity and enhances throughput. The automated analysis can be modified optionally by the user. The software gives comprehensive and accurate molecular calculations along with the R_f values. It has export, print and analysis report option which helps in saving the data for long term.

Statistical analysis

Attained data was analysed statistically by using IBM SPSS Statistics version 20 via applying One-way ANOVA and means were compared by applying Duncan's Multiple Range Test (DMRT) observed at the significance level $p \leq 0.05$.

Results

Growth parameters

Different growth parameters of two maize cultivars were checked under Cr stress. The parameters undertaken were monitored to asses' the alterations in plant growth were, number of leaves, plant height, root length, fresh and dry weight of root and shoot (Figures 1 and 2). The results were analysed and interpreted as follows.

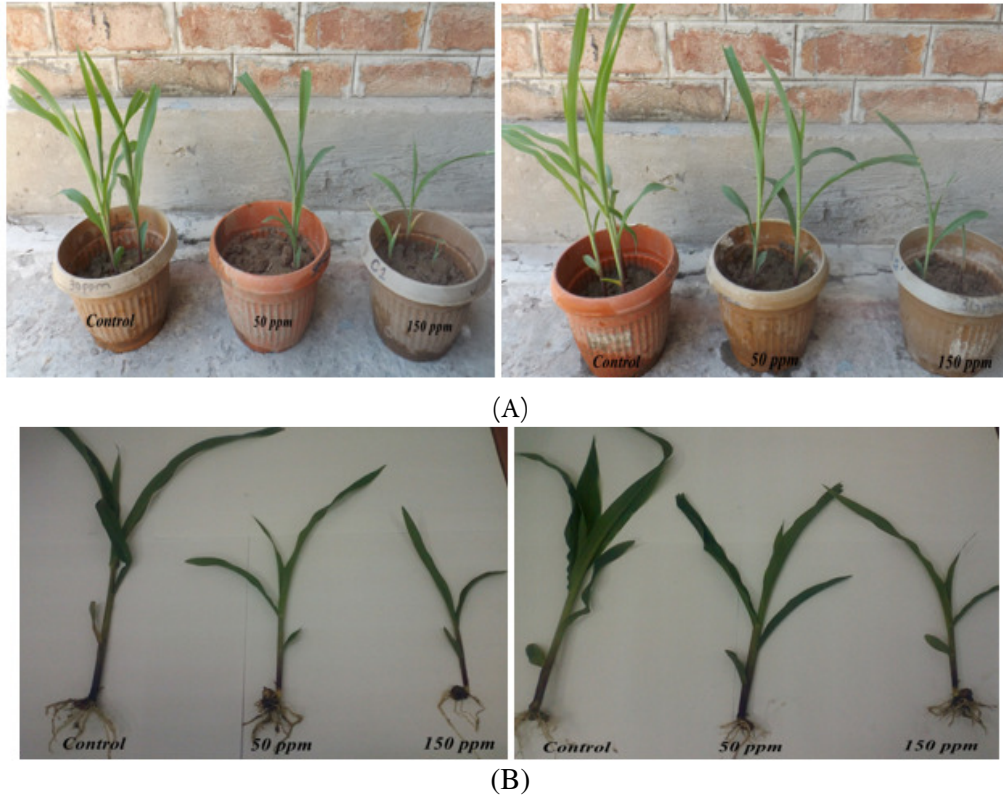


Figure 1. (A) Seedlings of maize cultivars C₁ (a), C₂ (b) taken for growth parameters subjected to different concentrations of Cr (0 ppm, 50 ppm, 150 ppm); (B) Maize cultivars C₁ (c), C₂ (d) subjected to different concentrations of Cr (0 ppm, 50 ppm, 150 ppm)

Number of leaves/plants

Data regarding the number of leaves per plant showed that the C₁ cultivar had shown 42% decline in number of leaves at 50 ppm and 64% at 150 ppm as compared to control. In C₂ cultivar the decline in the number of leaves per plant was 35% at 50 ppm and 65% in C₂ cultivar (Table 1).

Plant height

Growth of plant affected in response to Cr stress was visible in form of reduction in plant height. Decrease in plant height at 50 and 150 ppm was 18% and 35% respectively as compared to control in C₁. The decrease in C₂ cultivar was 20% in 50 ppm and 44% in 150 ppm as compared to control (Table 1).

Root length

Data revealed that root length decreased 33% at 50 ppm and 38% at 150 ppm in C₁ cultivar while C₂ cultivar has shown 25% decrease at 50 ppm and 43% at 150 ppm (Table 1).

Table 1. Effect of Cr supplied through soil on number of leaves, plant height and root length of maize cultivars

Treatments	No. of leaves/plant		Plant height (cm)		Root length (cm)	
	C ₁ Cultivar	C ₂ Cultivar	C ₁ Cultivar	C ₂ Cultivar	C ₁ Cultivar	C ₂ Cultivar
Control	4.66±0.57a	5.66±0.57a	12.02±0.50a	14.16±0.28a	7.23±0.25a	8.00±0.50a
50 ppm	2.66±0.57b	3.66±0.57b	9.83±0.28b	11.3±1.04b	4.83±0.28b	6.09±0.20b
150 ppm	1.66±0.57c	1.97±0.57c	7.76±0.25c	7.83±0.76c	4.83±0.28bc	4.50±0.50c

The lowercase letters in column showed the significant variations across treatments means at $p < 0.05$.

Shoot fresh weight

Results about shoot fresh weigh under the Cr stress are presented in Tale 2. Maximum effect of Cr was observed in 150 ppm in both cultivars. Shoot fresh weight decreased 49% at 50 ppm and 66% in 150 ppm in C₁ cultivar while in C₂ cultivar this decrease was 48% in 50 ppm and 71% in 150 ppm respectively as compared to control (Table 2).

Shoot dry weight

Shoot dry weight decreased 47% at 50 ppm and 76% in 150 ppm in C₁ cultivar. Shoot dry weight of C₂ variety decreased 33% at 50 ppm and 66% in 150 ppm as compared to control in C₂ cultivar (Table 2).

Table 2. Effect of Cr supplied through soil on shoot fresh and dry weight of maize cultivars

Treatments	Shoot fresh weight (g)		Shoot dry weight (g)	
	C ₁ Cultivar	C ₂ Cultivar	C ₁ Cultivar	C ₂ Cultivar
Control	2.76±0.51a	3.63±0.25 a	0.17±0.02a	0.21±0.02a
50 ppm	1.40±0.34b	1.86±0.11b	0.09±0.00b	0.14±0.10b
150 ppm	0.93±0.15c	1.02±.06c	0.04±0.01c	0.07±0.01c

The lowercase letters in column showed the significant variations across treatments means at $p < 0.05$.

Root fresh weight

There was a marked decrease in fresh weight of root from control to 150 ppm Table 3. Root fresh weight decreased 18% at 50 ppm and 75% in 150 ppm in C₁ cultivar while this decrease was 33% at 50 ppm and 57% at 150 ppm as compared to control in C₂ cultivar.

Root dry weight

Root dry weight increased 21% at 50 ppm and then showed a decrease of 60% at 150 ppm in C₁ cultivar. In C₂ cultivar an increase in dry weight of root was also observed at 50 ppm. It was observed about 56% increase at 50 ppm and then decreased 72% at 150 ppm as compared to control (Table 3).

Seed treatment

The morphological changes were visible in seeds of C₁ (Figure 2) and C₂ (Figure 3) cultivars subjected to different concentration of Cr (0, 50, 150 ppm) (Figures 3-4).

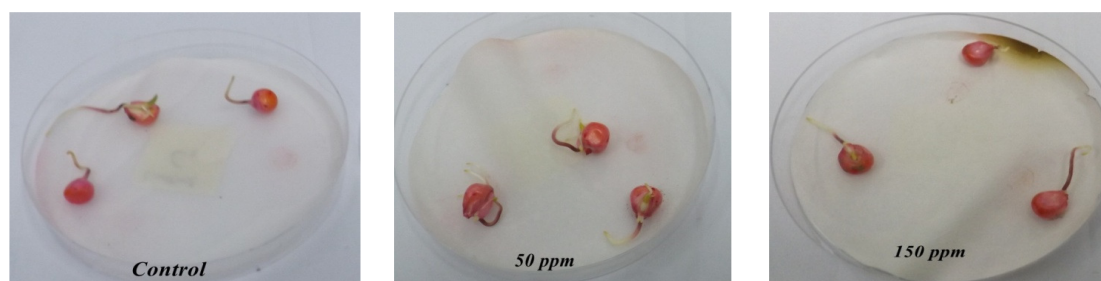


Figure 2. Seeds of C₁ cultivar subjected to different concentrations of Cr (0, 50, 150 ppm) for three days

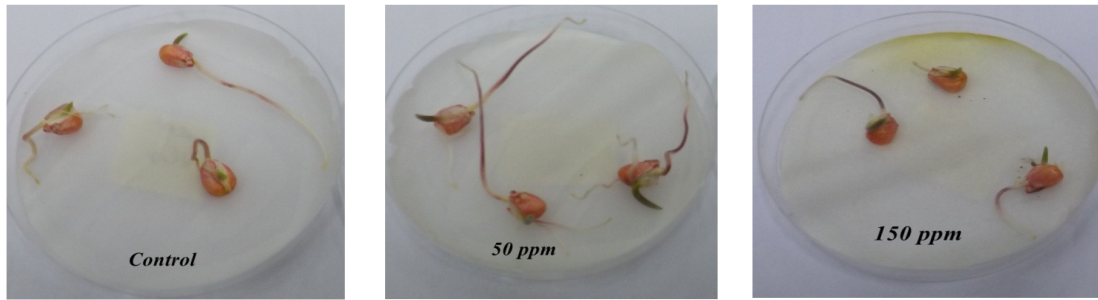


Figure 3. Seeds of C_2 cultivar subjected to different concentrations of Cr (0, 50, 150 ppm) for three days

Table 3. Effect of Cr supplied through soil on root fresh and dry weight of maize cultivars

Treatments	Root fresh weight (g)		Root dry weight (g)	
	C_1 Cultivar	C_2 Cultivar	C_1 Cultivar	C_2 Cultivar
Control	$1.22 \pm 0.19a$	$1.83 \pm 0.05a$	$0.17 \pm 0.02a$	$0.21 \pm 0.02a$
50 ppm	$1.0 \pm 0.14b$	$1.22 \pm 0.37b$	$0.09 \pm 0.06b$	$0.14 \pm 0.10b$
150 ppm	$0.30 \pm 0.46c$	$0.77 \pm 0.15c$	$0.04 \pm 0.01c$	$0.07 \pm 0.01c$

The lowercase letters in column showed the significant variations across treatments means at $p < 0.05$.

Protein quantification

Bradford Assay was carried out to measure concentration of total proteins in plant tissue lysates. For low concentrations (1-10) μg , micro-assay was performed whereas for high concentrations (10-100) μg , macro-assay was being performed. Standard curve using Bovine Serum Albumin was drawn for each case. The values of concentration of standards and their respective optical densities are given in the following Table 4.

Protein separation through sodium dodecyl polyacrylamide gel electrophoresis

SDS-PAGE of leaves, shoot and seeds of C_1 and C_2 cultivar was performed to examine the protein profile alterations in chromium treated seedlings. An un-stained protein marker of molecular weight ranging from 10-200 kDa (Fermentas life sciences) was used as reference to determine the molecular weight of polypeptide bands.

Protein expression in seeds of C_1 cultivar

Various expression in cultivars were found in seeds against induced chromium stress. These variations were found at molecular masses that were 136, 130, 115, 104, 85, 70, 57, 55, 41, 30, 25, 20, 17, 15 and 12 kDa (Figure 4).

Table 4. Protein profile alterations in seeds of C₁ cultivar in terms of fold change

Sr. No.	Mol. Wt (kDa)	Rf value	Expression in concentration of total protein load per sample (10 µg)				
			Control	50 ppm	Fold Change	150 ppm	Fold Change
1	136	0.076	0.76	-	-0.76	-	-0.76
2	130	0.087	-	0.82	+0.82	0.88	+0.88
3	115	0.111	0.58	-	-0.58	-	-0.58
4	104	0.142	-	0.41	+0.41	0.56	+0.56
5	85	0.192	0.75	-	-0.75	0.69	-0.92
6	70	0.240	0.56	0.58	+1.03	0.83	+1.48
7	57	0.310	0.62	0.66	+1.06	-	-0.62
8	55	0.352	0.55	0.75	+1.36	0.76	+1.38
9	41	0.450	1.38	2.20	+1.59	1.55	+1.1
10	30	0.555	0.89	-	-0.89	-	-0.89
11	25	0.625	-	1.15	+1.15	1.26	+1.26
12	20	0.680	0.81	0.83	+1.02	0.84	+1.03

+ sign indicate an increase and minus sign indicate a decrease in expression as compared to reference gel

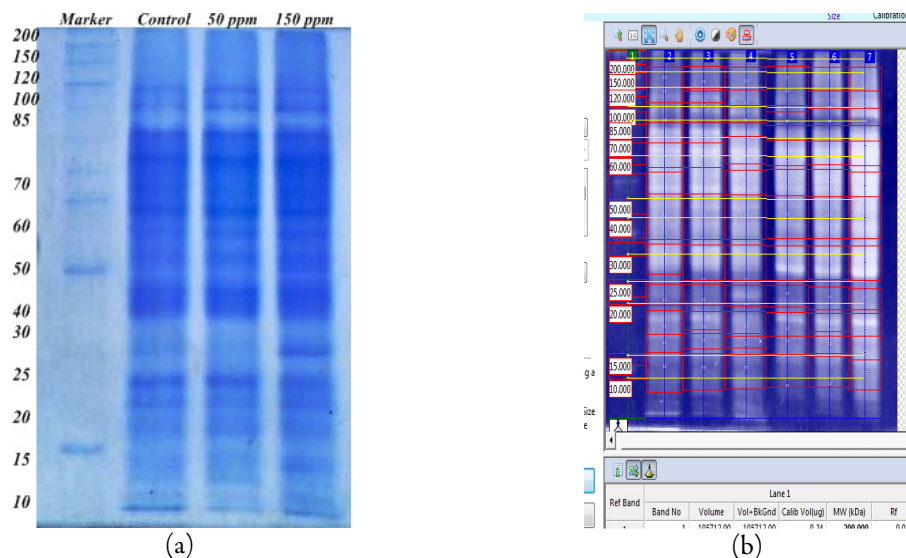


Figure 4. (a) SDS-PAGE of C₁ variety seeds (b) Gel image analysis of C₁ cultivar seeds through software (Image Quant TL)

Quantification of proteins

Gel analysis using image Quant TL software showed the difference in control, 50 ppm and 150 ppm concentrations in Table 4. Protein of 136 kDa expressed 0.76 µg in control, and disappeared in 50 and 150 ppm. Protein of 130 kDa was not detected in control but appeared in 50 and 150 ppm. It expressed 0.82 µg in 50 ppm, 0.88 µg in 150 ppm. Proteins like 115 kDa appeared only in control and its appearance was not detected in 50 and 150 ppm. Similarly, a 104 kDa protein was appeared to be induced in 50 and 150 ppm. Protein at 85 kDa expressed concentration 0.75 µg in control and 0.50 µg in 150 ppm. A protein of 70 kDa had expressed concentration of 0.56 µg in control, 0.58 µg in 50 ppm and 0.83 µg in 150 ppm. Protein of 57 kDa expressed 0.62 µg in control, 0.66 µg in 50 ppm and disappeared in 150 ppm. The concentration expressed by 41 kDa protein was 1.38 µg in control, 2.20 µg in 50 ppm and 1.55 µg in 150 ppm. Protein of 30 kDa had only shown expression in control and disappeared in 50 and 150 ppm. A protein of 25 kDa was appeared in 50 and 150 ppm. A protein of 20 kDa had expressed 0.81 µg in control, 0.83 µg in 50 ppm and 0.84 µg concentration

in 150 ppm. Some proteins showed minor changes in expression like 17 and 15 kDa proteins. A protein of 12 kDa had expressed concentration of 1.64 μg in control, 1.43 μg in 50 ppm and 1.38 μg in 150 ppm.

Protein expression in seeds of C₂ cultivar

The variation was found at molecular masses that were 141, 138, 120, 115, 100, 85, 65, 57, 55, 41, 30, 20, 17, 15 and 12 kDa (Figure 5).

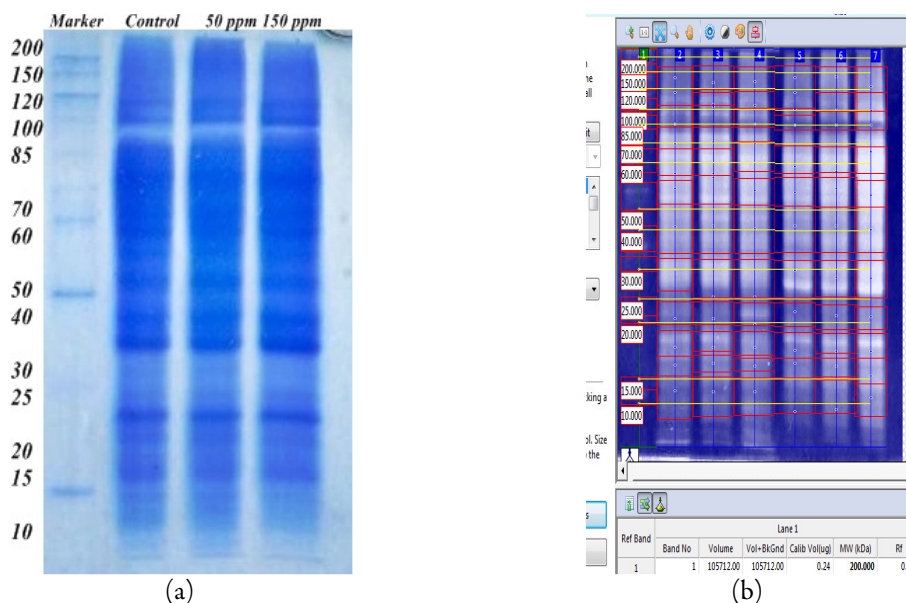


Figure 5. (a) SDS-PAGE of C₂ variety seeds (b) Gel image analysis of C₂ cultivar seeds through software (Image Quant TL)

Quantifications of proteins

Gel analysis using image Quant TL software showed the difference in control, 50 ppm and 150 ppm concentrations in Table 5. Protein of 141 kDa appeared in 50 ppm only and disappeared in 50 and 150 ppm. Protein of 138 kDa was only appeared in control and disappeared in response to stress in 50 and 150 ppm. A protein of 120 kDa appeared to be induced in response to 150 ppm. Protein of 115 kDa appeared in control only and its appearance was not detected in 50 and 150 ppm. A protein of 100 kDa expressed 0.24 μg in control, 0.66 μg in 50 ppm and 0.63 μg in 150 ppm. The proteins like 85 kDa presented minor changes in expression in all the three treatments. A protein of 65 kDa appeared to be induced in response to 150 ppm only. Protein of 57 kDa had expressed 0.76 μg in control, 1.40 μg in 50 ppm and disappeared in 150 ppm. Proteins like 55 and 41 kDa presented minor changes in expression. A protein of 30 kDa expressed concentration of 1.1 μg in control, 1.15 μg in 50 ppm and 1.09 μg in 150 ppm. The concentration expressed by 20 kDa protein was 0.88 μg in control, 0.93 μg in 50 ppm and 0.88 μg in 150 ppm. A protein of 17 kDa expressed 0.82 μg in control, 0.66 μg in 50 ppm and 0.81 μg in 150 ppm. Proteins of 15 and 12 kDa presented minor changes in expression in control, 50, and 150 ppm. A protein of 12 kDa had shown concentration of 1.28 μg in control, 1.27 μg in 50 ppm and 1.3 μg in 150 ppm.

Table 5. Protein profile alterations in seeds of C_2 cultivar in terms of fold change

Sr. No.	Mol. Wt (kDa)	Rf value	Expression in concentration of total protein load per sample (10 μ g)				
			Control	50 ppm	Fold Change	150 ppm	Fold Change
1	141	0.070	-	0.87	+0.87	-	
2	138	0.074	0.8	-	-0.8	-	-0.8
3	120	0.096	-	-	-	0.96	+0.96
4	115	0.111	0.64	-	-0.64	-	-0.64
5	100	0.155	0.24	0.66	+2.75	0.63	+2.63
6	85	0.192	0.55	0.72	+1.31	0.68	+1.23
7	65	0.259	-	-	-	0.64	+0.64
8	57	0.310	0.76	1.40	+1.84	-	-0.76
9	55	0.352	0.82	0.89	+1.09	0.69	-0.84
10	41	0.450	1.45	1.48	+1.0	1.36	-0.97
11	30	0.555	1.1	1.15	+1.05	1.09	-0.10
12	20	0.680	0.88	0.93	+1.06	0.88	0.00
13	17	0.753	0.82	0.66	-0.80	0.81	-0.98
14	15	0.780	0.66	0.65	-0.98	0.95	+1.43
15	12	0.820	1.28	1.27	-0.99	1.3	+1.01

+ sign indicate an increase and minus sign indicate a decrease in expression as compared to reference gel

Protein expression in shoot of C_1 cultivar

The variation was found at molecular masses that were 162, 154, 140, 115, 80, 70, 65, 60, 53, 41, 35, 30, 24, 20, 17, and 15 kDa (Figure 6).

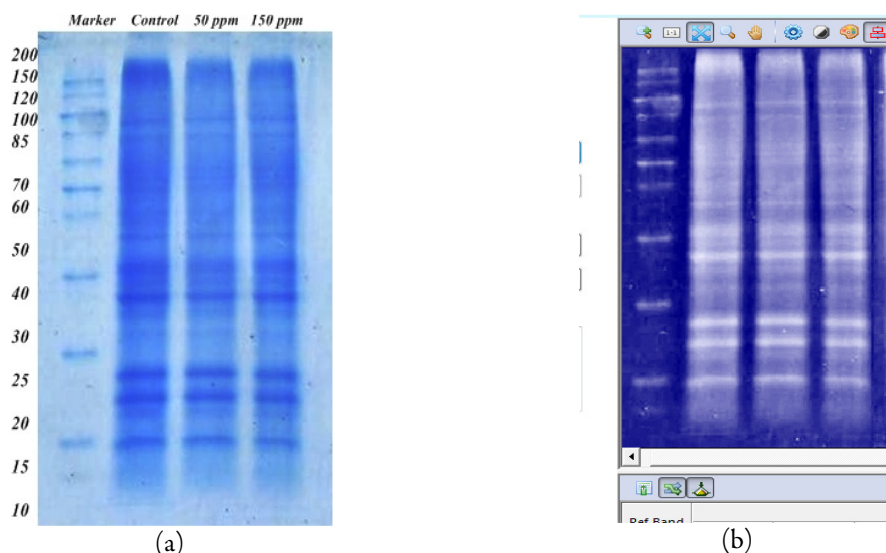


Figure 6. (a) SDS-PAGE of C_1 cultivar of shoot (b) Gel image analysis of C_1 cultivar shoot through software (Image Quant TL)

Quantifications of proteins

Gel analysis using image Quant TL software showed the difference in control, 50 ppm and 150 ppm concentrations in Table 6. Results showed that protein of 162 kDa expressed minor changes in all treatments. Protein of 154 kDa was expressed in 50 ppm only. The concentration expressed by 140 kDa protein was 0.53

μg in control, 0.71 μg in 150 ppm and absent in 50 ppm. Protein of 115 kDa only expressed in control and was not detected in 50 and 150 ppm. Protein of 80 kDa expressed concentration of 1.5 μg in control, absent in 50 ppm and reappeared in 150 ppm. A protein of 70 kDa was detected in control and 50 ppm, while it was not detected in 150 ppm. Proteins at 65 kDa was induced in 50 and 150 ppm and was absent in control. A protein of 60 kDa was detected only in 50 and 150 ppm. Protein of 53 kDa expressed in control and 150 ppm with concentration of 0.7 μg and 1.0 μg respectively. Protein of 41 kDa expressed minor changes in concentration in control, 50 and 150 ppm. A protein of 35 kDa expressed concentration of 0.51 μg in control, 0.57 μg in 50 ppm and 0.46 μg in 150 ppm. Protein of 30 kDa expressed almost same concentration in control, 50 ppm and 150 ppm. Protein of 24 kDa showed 0.93 μg in control, 2.0 μg in 50 ppm and 1.04 μg in 150 ppm. Protein of 20 kDa expressed 0.93 μg in control, 1 μg in 50 ppm, 1.04 μg concentration in 150 ppm. Protein of 17 kDa was induced in 50 and 150 ppm. The concentration expressed by 15 kDa protein was 2.09 μg in control, 1.16 μg in 50 ppm and 1.18 μg in 150 ppm.

Table 6. Protein profile alterations in shoot of C_1 cultivar in terms of fold change

Sr. No.	Mol. Wt (kDa)	Rf value	Expression in concentration of total protein load per sample (10 μg)				
			Control	50 ppm	Fold Change	150 ppm	Fold Change
1	162	0.083	0.69	0.82	+1.18	0.92	+1.33
2	154	0.092	-	0.92	+0.92	-	0.00
3	140	0.115	0.53	-	-0.53	0.71	+1.33
4	115	0.160	0.40	-	-0.40	-	-0.40
5	80	0.270	1.61	-	-1.61	0.58	-0.36
6	70	0.300	1.5	0.64	-0.42	-	-1.5
7	65	0.350	-	0.49	+0.49	0.84	+0.84
8	60	0.370	-	0.7	+0.7	1	+1
9	53	0.382	0.53	-	-0.53	0.40	-0.75
10	41	0.406	0.99	0.60	-0.60	0.67	-0.67
11	35	0.480	0.51	0.57	+1.11	0.46	-0.90
12	30	0.500	0.64	0.72	+1.12	0.73	1.14
13	24	0.635	0.93	2.0	+2.15	1.04	+1.11
14	20	0.710	0.57	0.64	+1.12	0.79	+1.38
15	17	0.765	-	0.73	+0.73	0.67	+0.67
16	15	0.800	2.09	1.16	-0.55	1.18	-0.56

+ sign indicate an increase and minus sign indicate a decrease in expression as compared to reference gel

Protein expression in shoot of C2 variety

The variations were found at molecular masses that were 157, 126, 115, 80, 70, 60, 46, 35, 30, 24, 20, 17 and 12 kDa (Figure 7).

Quantifications of proteins

Gel analysis using image Quant TL software showed the percentage volume difference in control, 50 ppm and 150 ppm concentrations in Table 7. Protein of 157 kDa was expressed in 1.01 μg concentration in control, 1.3 μg in 50 ppm and disappeared in 150 ppm. Similarly, protein at 126 kDa expressed 0.63 μg in control, 1.59 μg in 50 ppm and 0.80 μg in 150 ppm. Protein of 115 kDa expressed minor changes in concentration in all the three treatments. Protein of 80 kDa had shown expression of 0.87 μg in control, 0.59 μg in 50 ppm and 2.49 μg concentration in 150 ppm. A protein of 70 kDa induced in 50 and 100 ppm, while it was not detected in 150 ppm. Protein of 60 kDa was expressed 0.95 μg in control and 0.85 μg in 50 ppm only. The concentration expressed by 46 kDa protein was 2.04 μg in control, 0.47 μg in 50 ppm and 1.16 μg in 150 ppm. A protein of 41 kDa was induced in 150 ppm, it was not detected in control and 50 ppm. Concentration expressed by 35 kDa protein was 0.61 μg in control, absent in 50 ppm and 150 ppm. Some proteins showed

minor changes in expression like proteins of 30 and 24 kDa. The concentration expressed by 20 kDa protein was 0.82 μg in control, 0.78 μg in 50 ppm, and 0.80 μg in 150 ppm. Protein of 17 kDa had also shown minor changes in expression in all the three concentrations. Protein of 12 kDa expressed 1.11 μg in control, 0.68 μg in 50 ppm and 0.65 μg in 150 ppm.

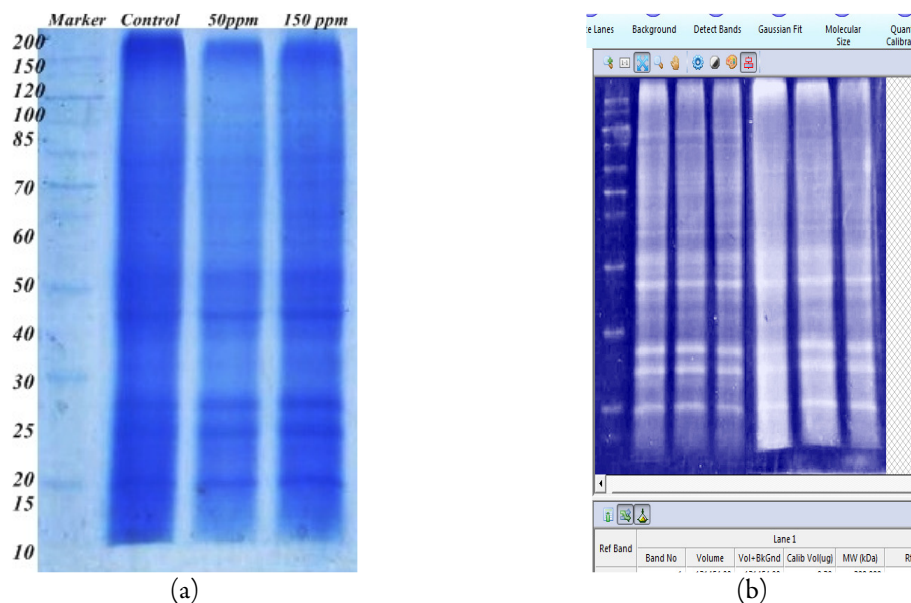


Figure 7. (a) SDS-PAGE of C_2 cultivar shoot (b) Gel image analysis of C_2 cultivar shoot through software (Image Quant TL)

Table 7. Protein profile alterations in shoot of C_2 cultivar in terms of fold change

Sr No.	Mol. Wt (kDa)	Rf value	Expression in concentration of total protein load per sample (10 μg)				
			Control	50 ppm	Fold Change	150 ppm	Fold Change
1	157	0.089	1.01	1.3	+1.28	-	-1.01
2	126	0.140	0.63	1.59	+2.52	0.80	+1.27
3	115	0.160	0.60	0.81	+1.35	0.86	+1.43
4	80	0.270	0.87	0.59	-0.67	2.49	+2.83
5	70	0.330	-	0.59	+0.59	0.50	0.50
6	60	0.370	0.95	0.85	-0.84	-	-0.95
7	46	0.394	2.04	0.47	-0.23	1.16	-0.56
8	41	0.406	-	0.67	+0.67	-	0.00
9	35	0.480	0.61	-	-0.61	-	-0.61
10	30	0.500	0.61	-	-0.61	0.75	+1.23
11	24	0.635	1.08	1.02	-0.94	2.04	+1.88
12	20	0.710	0.82	0.78	-0.95	0.80	-0.97
13	17	0.765	0.75	0.66	-0.88	0.64	-0.85
14	12	0.846	1.11	0.68	-0.61	0.65	-0.59

+ sign indicate an increase and minus sign indicate a decrease in expression as compared to reference gel

Protein expression in leaves of C_1 cultivar

Alterations in protein expression of maize leaves were observed. Intensity of protein bands varied against the stress induced using varied concentrations of chromium. These variations were found at molecular masses that were 140, 100, 70, 52, 46, 15 and 10 kDa (Figure 8).

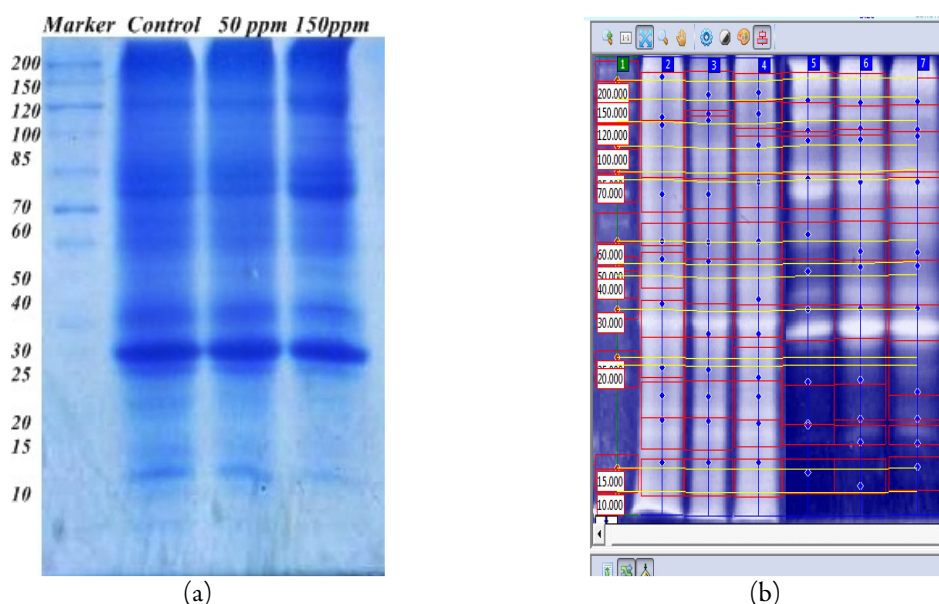


Figure 8. (a) SDS-PAGE of C1 variety leaves (b) Gel image analysis of C₁ cultivar leaves through software (Image Quant TL)

Quantifications of proteins

Gel analysis using image Quant TL software version 1.1 showed the difference in concentration in control, 50 ppm and 150 ppm concentrations in Table 8. Protein with 144 kDa expressed 0.57 μ g concentration in control and absent in 50 and 150 ppm. Similarly, proteins of 140 kDa was expressed 0.63 μ g in 50 ppm and 2.55 μ g in 150 ppm while not detected in control. Protein of 110 kDa showed 0.60 μ g concentration in control, 0.51 μ g in 50 ppm and 2.00 μ g in 150 ppm. Some proteins presented minor changes in expression like protein of 100 and 52 kDa. A protein of 70 kDa expressed 0.69 μ g concentration in control, 0.71 μ g in 50 and 2.05 in 150. A protein of 46 kDa showed 0.88 μ g concentration in control, 2.03 μ g in 50 ppm, and 0.75 μ g in 150 ppm. The protein of 30 kDa showed concentration 2.26 μ g, 0.67 μ g and 0.71 μ g in control, 50 and 150 ppm. Proteins like 15 kDa and 10 kDa showed minor changes in expression.

Table 8. Protein profile alterations in leaves of C₁ cultivar in terms of fold change

Sr No.	Mol. Wt (kDa)	Rf value	Expression in concentration of total protein load per sample (10 μ g)				
			Control	50 ppm	Fold change	150 ppm	Fold change
1	144	0.097	0.57	-	-0.57	-	-0.57
2	140	0.102	-	0.63	+0.63	2.55	+2.55
3	110	0.165	0.60	0.51	-0.85	2.00	+3.33
4	100	0.190	0.83	0.80	-0.96	1.0	+1.20
5	70	0.270	0.69	0.71	+1.02	2.05	+2.98
6	52	0.404	0.92	0.96	+1.02	0.93	-1.01
7	46	0.462	0.88	2.03	+2.30	0.75	-0.85
8	30	0.550	2.26	0.67	-3.37	0.60	-3.76
9	15	0.712	1.35	1.38	+1.02	-	-1.35
10	10	0.807	1.90	1.35	-0.71	0.10	-0.052

+ sign indicate an increase and minus sign indicate a decrease in expression as compared to reference gel

Protein expression in leaves of C₂ cultivar

The variations were found at molecular masses that were 180, 165, 130, 124, 117, 100, 85, 70, 55, 52, 30, 25, 18 and 10 kDa (Figure 9).

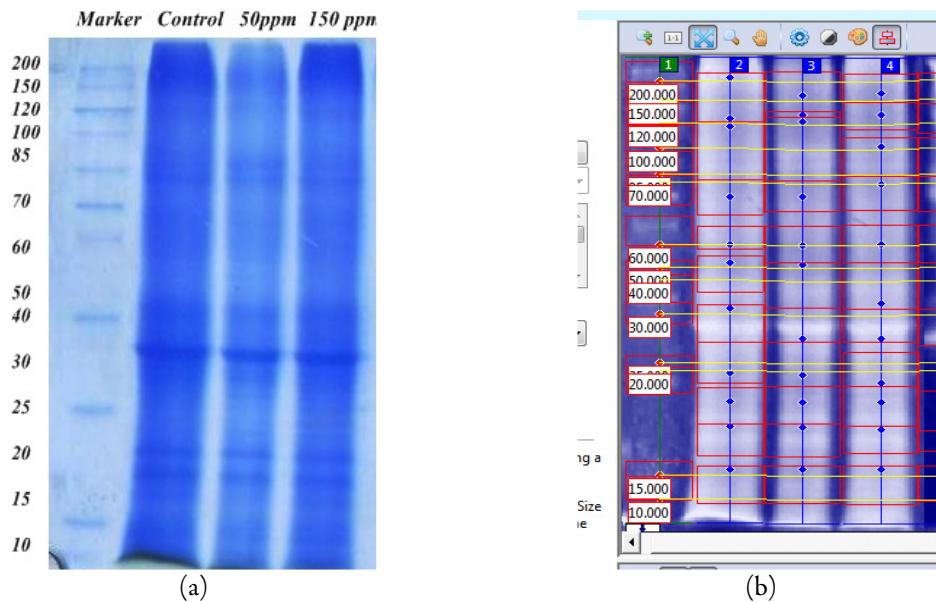


Figure 9. (a) SDS-PAGE of C₂ cultivar leaves (b) Gel image analysis of C2 variety leaves through software (Image Quant TL)

Quantifications of proteins

Gel analysis using image Quant TL version 1.1 software showed the percentage volume difference in control, 50 ppm and 150 ppm concentrations in Table 9. The protein of 180 kDa protein showed 0.66 µg concentration in control, 0.89 µg in 150 ppm and was absent in 50 ppm. A protein of 165 kDa expressed 0.74 µg concentration in 50 ppm and 0.78 µg in 150 ppm while it was not detected in control. Similarly, a protein of 130 kDa was expressed 0.16 µg in control, 0.78 µg in 50 ppm, 0.46 µg in 150 ppm. A protein of 124 kDa was detected in control, 50 and 150 ppm with the concentration of 0.60 µg, 0.49 µg and 1.59 µg respectively. Similarly, proteins of 100 kDa were detected in control, 50 and 150 ppm with the concentration of 2.01 µg, 0.90 µg and 0.64 µg respectively. Protein of 70 kDa was expressed 0.95 µg in 150 ppm and was absent in control and 50 ppm. The concentration of 55 kDa protein was 1.81 µg in control, 0.65 µg in 50 ppm and 0.89 µg in 150 ppm. A protein of 52 kDa showed 1.03 µg concentration in 150 ppm. Protein of 30 kDa was induced in 50 and 150 ppm in response to stress. Some proteins have shown minor changes in expression like 25 and 18 kDa. Protein of 10 kDa expressed 1.02 µg concentration in control, 0.95 µg in 50 ppm and 0.96 µg in 150 ppm.

Table 9. Protein profile alterations in leaves of C₂ cultivar in terms of fold change

Sr. No.	Mol. Wt (kDa)	Rf value	Expression in concentration of total protein load per sample (10 µg)				
			Control	50 ppm	Fold change	150 ppm	Fold Change
1	180	0.045	0.66	-	-0.66	0.89	+1.34
2	165	0.080	-	0.74	+0.74	0.78	+0.78
3	130	0.121	0.16	0.78	+4.875	0.46	+2.87
4	124	0.135	0.6	0.49	-0.81	1.59	+2.65
5	117	0.150	1.53	-	-1.53	-	
6	100	0.190	-	-		1.06	+1.06
7	85	0.200	2.01	0.9	-0.44	0.64	-0.31
8	70	0.270	-	-		0.95	+0.95
9	55	0.299	1.81	0.65	-0.35	0.89	-0.49
10	52	0.404	0.59	0.92	+1.55	1.03	+1.74
11	30	0.550	-	1.95	+1.95	1.08	+1.08
12	25	0.605		0.78	+0.78	0.78	+0.78
13	18	0.682	1.07	1.14	+1.08	0.9	-0.84
14	10	0.807	1.02	0.95	-0.93	0.96	-0.94

+ sign indicate an increase and minus sign indicate a decrease in expression as compared to reference gel

Discussion

Increase in heavy metal concentration decreases plant vitality and inhibit its growth. These metals at a very high concentration can cause ultimate death of plant (Aldoobie and Beltagi, 2013). Some of the physiological processes severely affected by Cr stress, the most phytotoxic effect on plants is stunted growth, reduction in biomass (Hall, 2002). In our present research work, growth attributes such as number of leaves, height of plant, fresh and dry weight of root and shoot of two cultivars were studied for the variations caused due to Cr toxicity. The growth of both cultivars was suppressed under high Cr concentration. But C₂ cultivar was proved to be more tolerant as compared to C₁ cultivar because of the decreased percentage variation in number of leaves of plant. The reduction in plant leaves number has also been reported by Shanker *et al.* (2004) due to Cr toxicity. Similar findings in terms of number of leaves were also observed in the findings of current study.

Different concentrations of Cr have affected plant height i.e., the height of C₁ cultivar decreased 18% in 50 ppm and 35% in 150 ppm whereas C₂ cultivar plant height has decreased up to 20 and 40% in 50 and 150 ppm (Table 1) concentrations respectively comparable with the study of Gupta and Solanki (2008). This can be due to lesser transport of nutrients to upper parts of plant and more to lower parts like root resulting in decreased shoot length and increased root length. Ouzounidou *et al.* (1998) suggested that this decrease in number of leaves and plant height might be due to heavy metal inhibitory effect which causes chromosomal aberrations and abnormal cell division. Heavy metal directly affected the respiration process in shoot system and reduction in cell growth and proliferation (Maria and Tadeusz, 2005). Fresh weight of shoot showed 66 % decline at 150 ppm in C₁ cultivar and 71% in C₂ cultivar. The decrease in fresh weight of root was 75% at 150 ppm in C₁ and 57% in C₂ cultivar. Similar kind of decrease in fresh weight of shoot was found by Maiti *et al.*, (2012) with the Cr concentrations ranging from 0 to 300 ppm.

A significant decrease in dry biomass and length of both maize cultivars was also observed. The plant metabolism changes could affect the growth of the plant as it has been reported in crops like mungbean (Ghani, 2010). Proteome of an organism responds to a number of stresses by undergoing a number of reversible and irreversible changes in its metabolism (Cvjetko *et al.*, 2014). The SDS-PAGE has been effective in protein profiling of different plants affected due to heavy metal treatment in tobacco seedlings against zinc and

cadmium interactions (Klose, 2009; Stefanic *et al.*, 2012). The technique has proved to be easy and economical one in assessing genetic diversity of maize plants (Iqbal *et al.*, 2014).

A protein of 41 kDa was 1.59-fold up-regulated at 50 ppm seeds of C₁ cultivar. Through protein database, it can be closely related to a chloroplast protein of 41 kDa. It is a stem loop binding protein which particularly functions in metabolism of chloroplast ribosomal RNA and its expression are much higher in seedlings (Bollenbach *et al.*, 2009). In C₁ cultivar 136, 115, 85, and 30 kDa protein bands seem to be suppressed in response to Cr stress while 130, 104, and 25 kDa protein bands appeared to be induced in response to Cr stress. A protein of 100 kDa showed significant 2.7-fold upregulation in both low and high treatments of Cr in C₂ cultivar. The alteration in this protein is reported by Bibi *et al.*, (2009) in chickpea genotypes in response to water stress. Protein of 57 kDa was 1.84-fold upregulated at 50 ppm in C₂ cultivar. This protein is reported to be involved in catalytic activity of glycolytic pathway, confirmed by uniprot and reported by Terol *et al.* (2002). Protein of 138 and 115 kDa disappeared at 50 and 150 ppm in response to Cr stress while 141, 120 and 65 kDa protein bands seemed to be appeared in response to Cr. Similar kind of results were reported by Labra *et al.* (2006). He analysed the proteomic response of maize seedlings against varied concentrations of Cr (2-1500 ppm) subjected to 72 h duration of exposure. The change in protein bands intensity was visible from 50 ppm to 1500 ppm. Some of the bands completely disappeared in response to stress while some other induced at high concentrations in response to Cr.

Molecular weight bands of 154, 140, 115, 80, 70 and 53 kDa were suppressed in C₁ cultivar shoots while 154, 65, 60, 17 kDa bands were suppressed in shoots of C₂ cultivar. Protein bands of 70, 41 kDa in C₂ shoots have appeared in response to Cr stress. One protein is up-regulated while one is down-regulated in response to stress in shoot of C₁ cultivar. A protein band of 80 kDa was 1.61-fold down regulated at 50 ppm in C₁ cultivar shoot while it was 2-fold up-regulated in C₂ cultivar in response to Cr. A similar 80 kDa protein was reported by Rothschild *et al.* (1996) in maize. UDP-glucose, protein transglucosylase enzyme related to this protein catalyses the first step of protein-bound alpha-glucan synthesis in developing maize endosperm. A band of 24 kDa showed a significant 2.15 and 1.88-fold up-regulation at 50 ppm and 150 ppm treatments of C₁ and C₂ cultivars. Two proteins were up-regulated in response to Cr in C₂ cultivar which confirmed more proteomic modifications in C₂ cultivar shoots as compared to C₁ cultivar. Similar studies were reported by Gianazza *et al.* (2007) in *Lepidium sativum*. There was a significant increase in level of proteins ranging from 10-25 kDa in response to cadmium chloride in *Lepidium sativum*.

Alterations in protein profile in response to Cr were observed in both cultivars of maize. Some proteins showed up-regulation while others were down-regulated in response to stress. A total of four proteins were found to be up-regulated in response to stress in leaves of C₁ cultivar while one was found to be down-regulated whereas in C₂ cultivar four proteins showed up-regulation and no protein was down-regulated. Leaves of C₂ cultivar proteome demonstrated more vitality and vigor as compared to C₁. The expression of protein bands in leaves of 140 kDa in C₁ and 144, 117 kDa in C₂ cultivar were suppressed in response to Cr concentrations as compared to control. A protein of 140 kDa in C₁ cultivar showed 2.55-fold upregulation at 150 ppm. Through protein database it is speculated to be a chloroplast stromal protein involved in metabolism of sulphate reduction pathway. A protein of 110 kDa showed an upregulation of 3.33-fold as compared to control. Similar kind of variations was reported by Ahmed *et al.* (2010) in different inbred lines of wheat.

Some high molecular weight proteins of 120 kDa in rainfed lines of wheat were upregulated as compared to control conditions. Another protein of 70 kDa showed 2.98-fold up-regulations at 150 ppm of C₁ leaves. This protein can be co-related by database analysis to a heat shock protein of *HSP* gene family. Heat shock proteins are induced in response to different stresses. These proteins aid plants to survive adverse conditions (Schmitt *et al.*, 2007). A protein of 30 kDa C₁ variety showed 3.37-fold down-regulated at both 50 and 150 ppm leaves. These results are in line with Unni and Rao (2001) who demonstrated the decrease in intensity of bands ranging from 22 to 68 kDa in *Rhizobium* in response to an abiotic stress. A protein of 130 kDa was up-regulated 4.88-fold at 50 ppm and 2.87-fold up-regulated at 150 ppm in C₂ cultivar. A protein of 124 kDa in

C₂ cultivar was 2.65-fold up-regulated at 150 ppm. A protein of 52 kDa was 1.55-fold up-regulated at 50 and 150 ppm in leaves of C₂ cultivar. The results of our study are in concordance to Abbas and Fayed (2014). A major band of 52 kDa was detected which could be closely related to a major mesophyll protein band of 56 kDa of ribulose-1,5-bisphosphate carboxylase/oxygenase in maize (Bewley and Black, 1994). The proteins present in medium molecular weight range might be involved in glycolytic pathways (Okamoto *et al.*, 2004). The appearance of 100, 70 and 30 kDa in C₂ variety leaves and 46 kDa band in leaves of C₁ cultivars point towards the induction of some stress proteins in response to heavy metal stress. Similar proteins were identified by Beltagi (2008) in transgenic corn plants in response to salt stress.

Conclusions

SDS-PAGE was used to examine protein modulation in two maize cultivars in response to varying levels of Cr in different plant tissues (seed, leaf, and shoot). Heat shock proteins (70 kDa), which have been shown to be triggered by a variety of environmental conditions, were found to be among the highly up-regulated proteins. Other proteins (56 kDa) that were up-regulated were thought to be involved in photosynthesis. Differentially expressed proteins that are up-regulated or down-regulated after additional validation can be useful indicators in the response to Cr stress and other heavy metal reactions in general. Overall, research on proteomic alterations in various plant tissues of maize under induced chromium stress has the potential to contribute to multiple sustainable development goals (SDGs) by addressing the challenges of food security, human health, water contamination, climate change, and the preservation of terrestrial ecosystems. However, 2-DE combined with mass spectrometric analysis (MALDI-TOF/TOF) is recommended for future validation of this study.

Authors' Contributions

Conceptualization, R.K.K., QuZ, and R.S.; methodology, A.Z. and R.K.; software, R.S., S.A.R. I.M.A. and K.H.A.; validation, QuZ., R.K.K. and M.A.S.; formal analysis, A.Z. K.A., F.M. and A.Z.; investigation, R.S.; resources, R.S.; data curation, A.Z. K.A., F.M.; writing—original draft preparation, R.S., and I.M.A, writing—review and editing, R.S., S.A.R., and QuZ., supervision, R.S.; project administration, R.S. and QuZ; funding acquisition, K.H.A. All authors have read and agreed to the published version of the manuscript.” All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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