Chromium(VI)-Induced Alterations in 2-D Protein Profiles and Antioxidant Defence Systems of Barley Cultivars

Arpa Çeşitlerinin 2-D Protein Profilleri ve Antioksidant Savunma Sistemindeki Krom(VI) Teşvikli Değişimler

Research Article

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ABSTRACT

The effects of Cr(VI) stress (0, 75, 150 and 225 μM) on Cr accumulation, growth, δ-aminolevulinic acid dehydratase (ALAD) activity, photosynthetic pigments content, proline content, malondialdehyde (MDA) content, antioxidant enzymes activity and 2-D protein profile were investigated using Cr-tolerant (Zeynelağa) and Cr-sensitive (Orza-96) barley cultivars. Zeynelağa accumulated significantly higher Cr in shoots than Orza-96. Zeynelağa had a lower reduction in shoot dry weight, chlorophyll and carotenoid contents than Orza-96. Although Cr(VI) significantly reduced the ALAD activity, there was no consistent difference between ALAD activities of barley cultivars. Cr(VI) increased proline and MDA contents, but this effect was more pronounced in Orza-96. Cr(VI) stress caused an increase in the activities of superoxide dismutase (SOD), guaiacol peroxidase (POD), ascorbate peroxidase (APX) and catalase (CAT). There was, in general, a marked difference between two barley cultivars in the extent of increased antioxidant enzyme activity. The results of the present study indicated that Zeynelağa seems to have better defense mechanisms to Cr(VI) stress compared to Orza-96. On the other hand, protein profile analyzed by two-dimensional gel electrophoresis showed differential expression of 26 proteins.

Key Words
Barley, Cr(VI), Antioxidant defence, 2-D protein profile

ÖZET

Krom birikimi, büyüme, δ-aminolevülinik asit dehidrataz (ALAD) aktivitesi, fotosentetik pigment içeriği, prolin içeriği, malondialdehit (MDA) içeriği, antioksidant enzim aktivitesi ve 2-D protein profilleri üzerine Cr(VI) stresinin (0, 75, 150 ve 225 μM) etkisi Cr-toleranslı (Zeynelağa) ve Cr-hassas (Orza-96) arpa çeşitleri kullanılarak araştırılmıştır. Orza-96'ya göre Zeynelağa gövde dokuları önemli düzeyde daha yüksek Cr biriktirmiştir. Orza-96'ya göre Zeynelağa gövde kuru ağırlığı, klorofil ve karotenoid içeriği daha düşük azalma göstermiştir. Cr(VI), ALAD aktivitesini önemli düzeyde azaltmış olmasına rağmen, arpa çeşitlerinin ALAD aktiviteleri arasında tutarlı bir farklılık bulunmamıştır. Cr(VI) prolin ve MDA içeriğini arttırmış; fakat bu etki Orza-96'da daha belirgin olarak belirlenmiştir. Cr(VI) stresi süperoksit dismutaz (SOD), guaiakol peroksidaz (POD), askorbat peroksidaz (APX) ve katalaz (CAT) aktivitelerinde artışa neden olmuştur. Genel olarak, artan antioksidant enzim aktiviteleri açısından iki arpa çeşidi arasında belirgin farklılık belirlenmiştir. Bu araştırmanın sonuçları, Orza-96'ya göre Zeynelağa'nın Cr(VI) stresine karşı daha iyi savunma mekanizmalarına sahip olduğunu göstermektedir. Diğer taraftan, iki-yönlü jel elektroforezi ile analiz edilmiş protein profilleri 26 proteinin farklı şekilde eksprese edildiğini göstermiştir.

Anahtar Kelimeler
Arpa, Cr(VI), Antioksidant savunma, 2-D protein profili

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INTRODUCTION

Environmental release of different chromium (Cr) compounds, mostly in the trivalent Cr(III) and hexavalent Cr(VI) forms, occurs mainly because of the widespread use of this metal in various industries [1]. Cr(VI), the most toxic form, exists as oxy anions such as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) [2]. Even though some crops are not affected by low concentrations of Cr (3.8 X 10^{-4} μM) [3], the metal is toxic at higher concentrations to most of the higher plants [4]. The toxicity of Cr(VI) results directly from its oxidative properties, as well as indirectly by the formation of free radicals during the reduction of Cr(VI) to Cr(III) inside the cell [5].

Inhibition of chlorophyll biosynthesis [6], as well as an increase in the level of chlorophyll has been reported in different plant species exposed to Cr(VI) [7]. However, impaired chlorophyll biosynthesis resulted in reduced total chlorophyll content as Cr-induced primary phytotoxic effects [8]. Proline, which occurs widely in higher plants, normally accumulates in large quantities in response to Cr(VI) stress [9]. Chromium has been reported to stimulate the formation of reactive oxygen species (ROS) causing oxidative stress and increased lipid peroxidation [1]. Since lipid peroxidation is ascribed to oxidative damage, measurement of malondialdehyde (MDA) levels, a common product of lipid peroxidation, is routinely used as sensitive index of oxidative stress [10]. The various antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (POD) scavenge ROS under Cr(VI) stress [11]. At the cellular level, plants have a range of potential mechanisms that might be involved in detoxification and tolerance of heavy metal stress. Rather than induced proteins that can tolerate the effects of heavy metals, these mechanisms all appear to be involved primarily in preventing the build-up of toxic concentrations at sensitive sites within the cell [12].

Little is known about the effect of Cr(VI) on the physiological and biochemical responses of Cr-tolerant and Cr-sensitive genotypes. Therefore, the present study was undertaken to assess antioxidant defence systems and 2-D protein profiles in leaves of barley cultivars exposed to Cr(VI) stress.

MATERIALS AND METHODS

Plant materials, growth and treatment conditions

Two barley (***Hordeum vulgare*** L.) cultivars were used in this study, Zeynelağa, which is relatively Cr(VI)-tolerant and Orza-96 which is a relatively Cr-sensitive [13]. The selected seeds were sterilized by rinsing in 2% aqueous (v/v) sodium hypochlorite (NaOCl) solution for 20 min, washed three times with distilled water and then soaked in distilled water for 3 h. Seeds were germinated under dark conditions at 25±1 °C for 3 days on filter paper wetted with distilled water. Three-day-old etiolated seedlings were grown hydroponically in 1/2 Hewitt's nutrient solution. Chromium(VI) was added to the nutrient solution at concentrations of 0,75, 150 and 225 μM in the form of potassium dichromate ($K_2Cr_2O_7$). The nutrient solution was renewed every two days to facilitate aeration of the roots and to maintain the pH of nutrient solutions in a range of 6.4-6.5. Plants were grown at 25±1 °C, 60% humidity and at 260 μmol m^{-2} s^{-1} light intensity for a 12 h photoperiod in a controlled growth chamber (Climacell, Germany). The barley seedlings were harvested and analyzed at the 7th day of exposure to Cr(VI).

Determination of growth, Cr accumulation, ALAD activity and photosynthetic pigments

The dry weights of shoot tissues were determined after drying at 80°C for 48 h. The Cr accumulation in dried shoot tissues was determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer 2100 DV) after microwave (Berghof, Germany) digestion. The Cr accumulation was calculated as μg g^{-1} DW.

The extraction of δ-aminolevulinic acid dehydratase (ALAD) from the excised leaf tissue was carried out according to Naito et al. [14] as described in Vajpayee et al. [8]. The ALAD activity assay was performed according to the method of Schneider [15]. The concentration of porphobilinogen (PBG) was calculated according to Mauzerall and Granick [16]. In addition, protein concentration of leaf crude extract was

determined according to Bradford [17].

Total chlorophylls (Chl ***a*** + ***b***) and carotenoids in the leaves (100 mg) of Cr-treated and untreated control seedlings were extracted and estimated according to Wellburn [18].

Determination of proline content, lipid peroxidation and antioxidant enzyme activities

The proline content of fresh leaves was estimated spectrophotometrically following the ninhydrin method described by Bates et al. [19] with slight modification. The level of lipid peroxidation was measured by estimating malondialdehyde (MDA), a decomposition product of peroxidized polyunsaturated fatty acid component of membrane lipids, using thiobarbituric acid (TBA) as the reactive material [20].

The leaf tissues (500 mg) were homogenized in 5 mL of 50 mM potassium phosphate buffer, pH 7.0 containing 1 mM ethylene diaminetetraacetate (EDTA) and 1% (w/v) polyvinylpyrrolidone (PVP). The extract was then centrifuged at 4 °C for 20 min at 14000 rpm in a cooled centrifuge. This supernatant was used to measure the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), guaiacol peroxidase (POD) and catalase (CAT). The protein concentration of leaf crude extract was determined according to Bradford [17]. Activity of SOD was assayed by using the photochemical nitro blue tetrazolium (NBT) method. The assay was performed in terms of SOD's ability to inhibit reduction of NBT to form formazan by superoxide radical as described by Beauchamp and Fridovich [21]. Activity of POD was determined at 25 °C with guaiacol [22]. Activity of APX was measured by following the rate of hydrogen peroxide-dependent oxidation of ascorbic acid [23]. Finally, the activity of CAT was assayed following H_2O_2 decomposition according to Aebi et al. [24].

Protein extraction and 2-D electrophoresis

The first leaf tissues (500 mg) of control and 225 μM Cr(VI)-treated seedlings of barley cultivars were sampled and total protein extraction was performed using the procedure of Damerval et al. [25]. Protein concentration of each sample was determined using Bovine Serum Albumin (BSA) standard curve according to Ramagli and Rodriguez [26]. Total protein extracts were used for two-dimensional gel electrophoresis according to the method of Naqvi et al. [27]. The first dimension (IEF) was performed using rod gels [28]. After isoelectric focusing, the second dimension (SDS-PAGE) was carried out on 12% acrylamide gels according to Laemmli [29]. For silver staining, the 2D gels were fixed and silver stained using the procedure of Blum et al. [30]. Relative molecular weights (kDa) and isoelectric points of differentially induced-proteins in Cr(VI)-stressed seedlings were detected.

Statistical analysis

All the experiments were repeated at twice with three replicates (***n*** = 6). Statistical analysis was performed using SPSS statistical package version 16.0. A one-way ANOVA test was used to confirm the significance of the data. Comparison of all means was done by Duncan's multiple range test ($P < 0.05$).

RESULTS AND DISCUSSION

Effect of Cr(VI) on dry weight, Cr accumulation, ALAD activity and photosynthetic pigments

The present study showed that Cr(VI) stress markedly reduced dry weights of Cr-tolerant Zeynelağa (12-30% reduction) and Cr-sensitive Orza-96 (19-50% reduction) barley cultivar (Table 1), which is in agreement with previous findings that Cr(VI) inhibits plant growth in pea [11], green gram [31] and barley [32]. These results reflect Zeynelağa barley cultivar's tolerant nature. The decrease in growth might be attributed to the significant decrease in photosynthetic pigments observed in this study. A reduction of plant biomass under Cr(VI) stress might be a consequence of poor assimilation, poor production, translocation and/or partitioning of assimilates. In our studies, Cr accumulation increased with an increase in Cr(VI) concentration in Zeynelağa and Orza-96 barley cultivars (Table 1). At the highest Cr(VI) concentration, Zeynelağa accumulated 95 μg Cr g^{-1} DW in shoot, whereas Orza-96 accumulated 80 μg Cr g^{-1} DW. This result suggests that high Cr accumulation in the shoot cells of Zeynelağa may

Table 1. Cr accumulation, dry weights, ALAD activity and photosynthetic pigment contents in leaves of barley cultivars exposed to different Cr(VI) concentrations

Barley cultivars	Cr(VI) (mM)	Cr accumulation	Dry weight	ALAD activity	Total chlorophylls	Carotenoids
		mg g DW^{-1}	% of control			
Zeynelağa	0	0.61±0.2 a*	100.0±0.0 a	100.0±0.0 a	100.0±0.0 b	100.0±0.0 b
	75	16.3±1.1 c	88.0±1.6 b	79.6±1.6 b	115.2±3.9 a	111.7±2.3 a
	150	36.4±1.3 e	78.5±2.5 c	61.9±2.5 d	98.6±1.3 b	93.3±1.6 c
	225	95.3±2.1 g	69.6±1.7 d	50.2±1.6 e	95.5±1.2 c	76.1±2.2 e
Orza-96	0	0.58±0.1 a	100.0±0.0 a	100.0±0.0 a	100.0±0.0 b	100.0±0.0 b
	75	11.2±0.5 b	80.6±4.0 c	84.4±3.1 b	90.1±4.2 d	94.7±2.0 c
	150	33.2±2.3 d	59.1±3.6 e	75.6±1.6 c	83.1±1.7 e	82.7±1.7 d
	225	79.7±3.3 f	50.0±1.7 f	48.6±1.6 e	70.2±2.3 f	60.7±1.4 f

* Different letters indicate significantly different values according to Duncan's test ($P < 0.05$).

be a natural anti-toxicity response of this cultivar against Cr(VI) stress.

Inhibition of chlorophyll biosynthesis under Cr(VI) stress has been reported for the aquatic plant, *Nymphaea alba* L., [8], but there have not been reports about the effect of chromium on chlorophyll biosynthesis in terrestrial crop plants. In the present study, the reduction in total chlorophyll content was significant in Orza-96 in all Cr(VI) concentrations, while it was significant at only 225 μM Cr(VI) in Zeynelağa (Table 1). Although the reduction in chlorophyll content of Cr(VI)-treated seedlings might be attributed to reduced ALAD activity induced by Cr(VI) [8], it can also be related to membrane oxidative damage produced by the heavy metal-induced ROS generation. Reduced ALAD activity results in a lower availability of PBG required for chlorophyll biosynthesis [33]. Our results also show that ALAD activity decreased in both Zeynelağa and Orza-96. On the other hand, decreased chlorophyll content showed a positive correlation with reduced ALAD activity in case of Orza-96. However, this correlation was observed in Zeynelağa at only 225 μM Cr(VI). As Cr(VI) can replace Mg ions from the active sites of many enzymes [8], it may also reduce ALAD activity. In the present study,

Table 2. Proline contents, MDA contents and antioxidant enzyme activities in leaves of barley cultivars exposed to different Cr(VI) concentrations

Barley cultivars	Cr(VI) (mM)	Proline	MDA	SOD	APX	POD	CAT
		mg g FW^{-1}	mmol g FW^{-1}	U mg^{-1} protein	mmol min^{-1} mg^{-1} protein		
Zeynelağa	0	7.13±1.3 a*	24.1±1.1 a	12.3±0.4 a	0.44±0.02 a	5.84±0.7 a	2.94±0.08 b
	75	7.49±0.7 a	24.2±2.7 a	21.7±1.2 c	0.44±0.03 a	11.1±1.6 b	2.97±0.09 b
	150	12.9±0.6 b	29.8±1.3 b	22.5±1.3 c	0.75±0.03 c	40.4±2.3 d	3.68±0.09 d
	225	14.5±1.0 b	30.7±2.8 b	24.9±0.5 d	0.92±0.02 d	92.9±5.5 f	4.14±0.13 e
Orza-96	0	5.91±1.2 a	22.4±0.8 a	11.2±0.5 a	0.47±0.02 a	5.99±0.6 a	2.92±0.08 b
	75	7.86±1.7 a	36.8±1.6 c	13.9±0.8 b	0.65±0.01 b	6.60±0.5 a	2.59±0.13 a
	150	25.6±2.7 c	47.1±3.9 d	20.8±1.1 c	0.94±0.02 d	29.8±1.4 c	3.42±0.09 c
	225	35.7±3.2 d	66.9±2.3 e	33.8±1.6 e	1.59±0.04 e	55.7±3.0 e	4.07±0.12 e

* Different letters indicate significantly different values according to Duncan's test ($P < 0.05$).

carotenoid contents in both barley cultivars decreased with increasing Cr(VI) concentrations and this reduction was more pronounced in Orza-96. The carotenoid contents showed a positive correlation with chlorophyll contents. Decreased carotenoid content of Cr-sensitive Orza-96 may result in lesser availability of carotenoid required for protection of chlorophyll. Our results suggested that photosynthetic pigment content may be a potential marker for discriminating the Cr-tolerant cultivars rather than ALAD activity.

Effects of Cr(VI) on proline, lipid peroxidation and antioxidant enzymes

The amino acid proline is present in many plant species and normally accumulates in large quantities in response to Cr(VI) [9]. There was no significant difference in the proline content of Cr-tolerant Zeynelağa and Cr-sensitive Orza-96 barley cultivars at 75 μM Cr(VI) compared to their controls (Table 2). Zeynelağa showed about 1.8 and 2.0 times higher proline content in leaves compared to control at 150 and 225 μM Cr(VI) respectively. Under the same conditions, proline content of Orza-96 was 4.3 and 6.0 times higher than control. These results suggested that proline accumulation in leaves of Cr-treated barley cultivars may be a symptom of Cr toxicity rather than an indication of Cr tolerance.

Lipid peroxidation (measurement of MDA levels) is an indicator of the oxidative stress induced by ROS [34]. Protonation of the superoxide radical can produce the hydroxyl radical ($OH^{\cdot}$), which can convert fatty acids to toxic lipid peroxides, so destroying biological membranes [35]. In the present study, the MDA content in leaves of both barley cultivars increased markedly when the seedlings were exposed to Cr(VI) stress (Table 2). The increase in MDA content of both barley cultivars indicate increased levels of ROS production under Cr(VI) stress. Orza-96 had a higher MDA content than Zeynelağa in all Cr(VI) concentrations, indicating less oxidative damage and better stress abating tendency of Zeynelağa, which in turn accounted for the limited inhibition of growth.

Plant cells have antioxidant defense mechanisms for scavenging of ROS. These include enzymatic mechanisms involving antioxidant enzymes such as SOD, APX, POD and CAT [31, 35]. The present results showed that activities of SOD, APX, POD and CAT were increased in Zeynelağa and Orza-96 barley cultivars under Cr(VI) stress compared to controls, which are consistent with previous reports that Cr(VI) increase the activity of antioxidant enzymes [31, 32]. SOD, the first enzyme in the detoxifying process, catalyzes the dismutation of superoxide radicals ($O_2^{\cdot -}$) to H_2O_2 and O_2. Our data showed that SOD activities of both barley cultivars increased in the different Cr(VI) concentrations compared to their controls. In contrast to these results, Dixit et al. [36] observed that 200 μM Cr(VI) produced a significant inhibition in SOD activity of *Pisum sativum*. The SOD activity of Orza-96 was increased more than that of Zeynelağa at 225 μM Cr(VI) concentration. It seems that up-regulation of SOD in Orza-96 was not sufficient to detoxify the superoxide radicals completely and this leads to a lower tolerance towards Cr(VI) stress. H_2O_2, a product of SOD activity, is also toxic to cells and has to be further detoxified by APX, POD and CAT [35]. Peroxidase and CAT are potential scavengers of H_2O_2, which minimize its accumulation and diffusion across cell membranes, preventing peroxidative damage to cell constituents [37]. In our study, APX and POD activities increased significantly with increasing Cr(VI) concentrations in both barley cultivars. Increases in peroxidase activities suggested their role in the detoxification of H_2O_2 and their up-regulation under Cr-induced oxidative stress, as established previously with reference to Cr(VI) stress [31, 32]. In the present study, Zeynelağa had higher POD and lower APX activities than Orza-96. Among H_2O_2 destroying enzymes, the increase in CAT activity was lower than those of APX and POD. The reason for this could be that CAT is present only in the peroxisome and has low substrate affinities since it requires simultaneous access of two molecules of H_2O_2 [38]. We observed that the extent of antioxidant enzyme activity under Cr(VI) stress varies with cultivar. This suggests different antioxidant responses of barley cultivars under Cr(VI) stress. The potential cellular mechanisms in detoxification of heavy metals include their sequestration in the cell wall and vacuole, where the POD is especially localized [39]. Our results suggested that POD

Table 3. Protein polymorphism in leaves of barley cultivars in 225 μM Cr(VI) concentration compared to the control.

Protein no	MA (kDA)	pI	Barley cultivars	
			Zeynelağa	Orza-96
1	15.6	6.6	↓*	↓
2	15.6	7.0	↓	↓
3	21.0	6.6		↓
4	21.0	6.9	↑	↑
5	21.6	6.6	↓	↓
6	22.0	6.3	↓	↓
7	22.0	6.6	+	+
8	22.1	6.6		↓
9	22.1	6.9		↑
10	22.4	7.5	↑	↑
11	22.5	6.1	↑	
12	22.5	6.9	+	+
13	22.6	6.2		↑
14	22.8	7.5	↑	↑
15	23.0	5.6	↓	↓
16	23.0	7.0	↓	↓
17	23.0	7.1	↓	↓
18	23.1	6.3	↓	↓
19	23.3	7.0	↓	↓
20	23.4	7.4	↓	↓
21	23.9	6.3	↓	↓
22	23.5	6.6	↓	↓
23	23.9	7.1	↑	↑
24	25.2	5.8	↓	↓
25	25.2	6.3	↓	
26	30.1	5.7	↓	↓

* +: Newly synthesized protein, ↑: Up-regulated protein, ↓: Down-regulated protein

activity in Zeynelağa cultivar was more efficient in destroying H_2O_2 than APX and CAT under Cr(VI) stress. Higher POD activity may be an indicator of Cr(VI) tolerance in Zeynelağa that accumulated more Cr in shoot tissues. Similar results were also reported for Cd-tolerant maize cultivar exposed to Cd [40].

Effect of Cr(VI) on 2-D protein profiles

High resolution 2-D gel electrophoresis was used for better separation of proteins. Three independent experiments were performed; high reproducibility was obtained. Under 225 μM Cr(VI) stress, protein profile analyzed by 2-D gel electrophoresis showed differential expression of 26 proteins (Figure 1). The molecular masses and pI values of each protein are listed in Table 3. The synthesis of 5 proteins (21.0-23.9 kDa, pI 6.1-7.5) were up-regulated in Zeynelağa, while synthesis of 6 proteins (21.0-23.9 kDa, pI 6.2-7.5) were up-regulated in Orza-96. However, the amounts of 15 and 16 proteins (15.6-30.1 kDa, pI 5.7-7.0) were down-regulated in Zeynelağa and Orza-96, respectively. Interestingly two proteins (22.0 kDa, pI 6.6; protein no: 7 and 22.5 kDa, pI 6.9; protein no: 12) were newly synthesized in both barley cultivars under Cr(VI) stress. However, Labra et al. [41] detected only reproducible up-regulated proteins in maize subjected to Cr(VI). These proteins were classified as antioxidant enzymes, sugar metabolism and other stress-response proteins [41]. In this sense, the future identification of differentially expressed proteins related to Cr(VI) stress will be important to understand the biological processes involved in Cr(VI) tolerance.

In conclusion, although Cr content in the shoot tissue of Orza-96 was lower at all Cr(VI) concentrations, Cr(VI)-induced dry weight reduction was higher than that of Zeynelağa. Our results indicated that the chlorophyll, carotenoid, MDA and prolin contents in leaf tissues were more affected from Cr(VI) stress in Cr-sensitive Orza-

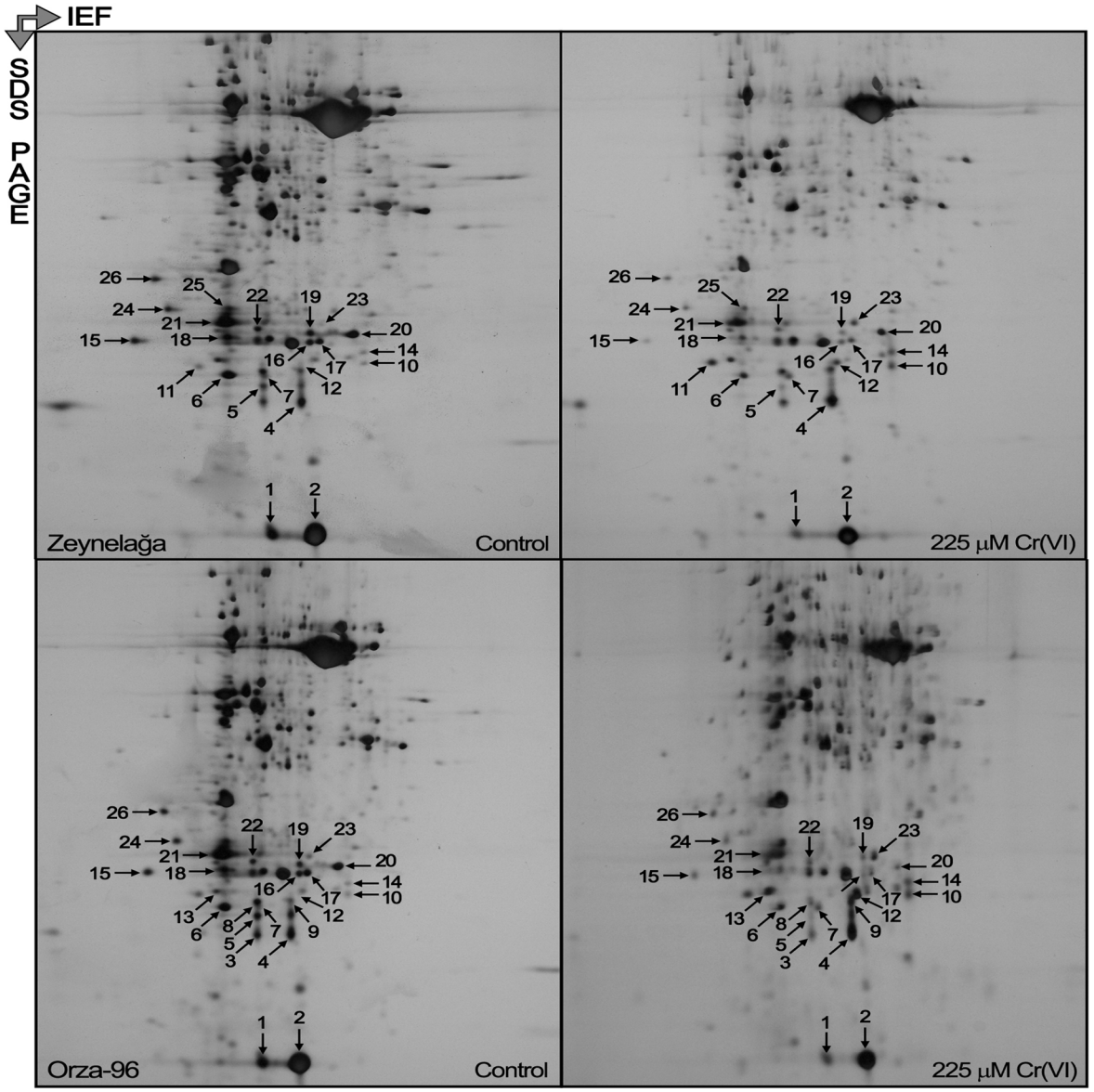

Figure 1. 2-D profiles of the soluble leaf proteins extracted from control and 225 μM Cr(VI)-treated seedlings of barley cultivars. Newly synthesized, up- and down-regulated proteins are indicated by arrow in control and 225 μM Cr(VI) gels (Table 3).

96 in comparison to Cr-tolerant Zeynelağa. These biochemical parameters could be effective criteria for discriminating between barley cultivars with differ in Cr(VI) tolerance. On the other hand, some protective mechanisms, such as activity of antioxidant enzymes, and especially of POD, might be more significant factors in tolerance. However, identification and characterization of Cr-responsive proteins will not only advance our understanding of Cr tolerance mechanisms, but more importantly, will also provide the new molecular information that researchers will use to develop and improve crop cultivars better suited for cultivation on contaminated soils.

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