

Effect of Cadmium on Growth and Concentrations of Cadmium, Ascorbic Acid and Sulphydryl Groups in Durum Wheat Cultivars

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Received: 18.03.2003

Abstract: By using two durum wheat cultivars (*Triticum durum* L. cvs. Balcali-85 and C-1252) a nutrient solution experiment was carried out to study i) genotypic variation in cadmium (Cd) tolerance, ii) Cd concentrations in plants, and iii) the role of ascorbic acid and non-protein SH groups (SH: sulphhydryl) in Cd tolerance. Plants were grown under controlled climatic conditions for 15 days and subjected to increasing Cd supply (0, 6, 30, 75 and 150 µM). Of the two cultivars, C-1252 showed greater sensitivity to Cd toxicity as judged from the severity of Cd toxicity symptoms on leaves. Increasing Cd supply markedly reduced the shoot and root dry weight of both cultivars, and these decreases were more marked in C-1252. Cd concentrations of plants were strongly increased by enhanced Cd supply, especially in the roots. C-1252 tended to have greater amounts of Cd in the shoots, but lower amounts in the roots than Balcali-85. Ascorbic acid concentrations in the roots and shoots were similarly affected by increasing Cd supply in both cultivars. SH concentrations were similar in the shoots of Balcali-85 and C-1252, and showed a slight increase due to Cd treatments. However, in the roots, Cd supply resulted in dramatic increases in concentrations in the SH groups, particularly in Cd-tolerant Balcali-85. The results indicate that SH-containing compounds (e.g., phytochelatins) and the retention of Cd in the roots are possibly involved in the expression of high Cd tolerance in durum wheat cultivars.

Key Words: cadmium, tolerance, durum wheat, glutathione, ascorbic acid

Kadmiyumun Makarnalık Buğday Çeşitlerinde Büyümeye ve Kadmiyum, Askorbik Asit ve Sülfidril Grupları Konsantrasyonuna Etkisi

Özet: Farklı 2 makarnalık buğday çeşidi kullanılarak (*Triticum durum*, Balcalı-85 ve Ç-1252) bir besin çözeltisi denemesi kuruldu ve bu denemeye makarnalık buğday çeşitlerinde i) kadmiyum (Cd) toleransındaki genotipsel farklılık, ii) Cd konsantrasyonu ve iii) askorbik asit ve non-protein SH-gruplarının (SH: sülfidril) Cd toleransındaki rolü araştırıldı. Bitkiler, kontrollü iklim koşullarında 15 gün süreyle artan dozlarda Cd (0, 6, 30, 75 ve 150 µM) uygulamaları altında yetiştirildi. Buğday çeşitlerinden Ç-1252'nin, Cd toksisitesinin yapraklardaki semptomlarının şiddeti bakımından Cd toksisitesine daha fazla duyarlı olduğu gözlenmiştir. Artan dozlarda uygulanan Cd, her iki çeşitte de yeşil aksam ve kök kuru madde üretimini önemli oranda azaltmıştır. Bu azalmaların C-1252'de, Balcalı-85'e göre daha belirgin olduğu saptanmıştır. Bitkilerin Cd konsantrasyonları Cd uygulamasıyla, özellikle kökte olmak üzere, çarpıcı biçimde artış göstermiştir. Çeşitlerden Ç-1252, Balcalı-85'e göre yeşil aksamda daha fazla Cd içerme eğilimi gösterirken, kökte daha az Cd'a sahip olduğu bulunmuştur. Bitkilerin askorbik asit konsantrasyonu, her iki çeşitte de Cd uygulamalarından benzer biçimde etkilenmiştir. Artan Cd uygulamaları ile yeşil aksamda SH-grupları konsantrasyonu her iki çeşitte de benzer biçimde az düzeyde bir artış göstermiştir. Ancak, köklerde artan Cd uygulaması ile SH-grupları konsantrasyonunda, özellikle Cd toksisitesine dayanıklı Balcalı-85 çeşidinde olmak üzere, çarpıcı düzeyde artışların olduğu saptanmıştır. Sonuçlar, makarnalık buğday çeşitlerinin farklı Cd toleransında SH içeren bileşiklerin (örneğin fitoşelatinlerin) ve köklerde Cd'un tutulmasının önemli bir role sahip olabileceğini göstermektedir.

Anahtar Sözcükler: kadmiyum, tolerans, makarnalık buğday, glutatyon, askorbik asit

Introduction

Cadmium accumulation in soils and crop plants is an increasing concern affecting human health and crop production (Wagner, 1993; Hall, 2002). Although Cd is

not an essential mineral nutrient for crop plants, it can be easily taken up by plant roots when the growth medium contains high levels of it. Increases in the Cd concentration of soils are generally caused by the

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application of sewage sludges and Cd-rich phosphate fertilizers, and mining (McLaughlin et al., 1999; Nolan et al., 2003). Crop plants greatly differ in their uptake and transport of Cd. Differences in Cd uptake and accumulation have been shown both among plant species (Guo et al., 1995; Grant et al., 1998; Cakmak et al., 2000a; Ozturk et al., 2003) and between genotypes of a given species (Clarke et al., 2002; Dunbar et al., 2003).

Plant mechanisms affecting the root uptake and shoot transport of Cd can also affect the expression of Cd toxicity in plants, and decreases in yield (Cakmak et al., 2000b; Kochian et al., 2002; Dunbar et al., 2003). Therefore, the selection of plant genotypes with high ability to repress root uptake and shoot transport of Cd is a reasonable approach to alleviate adverse effects of Cd toxicity in crop plants.

Despite similar Cd concentrations in the leaves or shoots, plant genotypes can differ in their tolerance to Cd toxicity. Cd, when taken up in cells, can be detoxified by Cd-binding proteins such as phytochelatins or metallothionins (Grant et al., 1998; Cobbett, 2000; Hall, 2002). The ability of plant genotypes to detoxify Cd by Cd-binding proteins can differ between and within plant species, and this plays a critical role in the expression of high tolerance to Cd toxicity. Glutathione (or non-protein SH groups) is directly involved in the synthesis of Cd-binding proteins and thus in the development of Cd tolerance in plants (Howden et al., 1995; Hall, 2002). Recently, Xiang et al. (2001) showed that plants with low levels of glutathione were highly sensitive to low levels of Cd in the growth medium due to the limited capacity of plants to synthesize phytochelatins. However, in the literature, there are controversial results concerning the role of phytochelatins in the Cd tolerance of plants (Stolt et al., 2003).

Glutathione together with ascorbic acid affects plant tolerance to reactive oxygen species (ROS) by participation in the detoxification of ROS in plant cells (Noctor and Foyer, 1998). There are several pieces of evidence showing that Cd toxicity represents an oxidative stress catalyzed by ROS (Dixit et al., 2001; Romero-Puertoas et al., 2002). The involvement of glutathione and ascorbic acid in the tolerance of plants to Cd toxicity has been shown in different plant species (El-Naggar and El-Sheekh, 1998; Wu and Zhang, 2002; Mendoza-Cozatl et al., 2002).

To our knowledge, there is no study in the literature dealing with Cd tolerance in durum wheat genotypes or the role of antioxidants, such as ascorbic acid and glutathione, in the expression of Cd toxicity in durum wheat. In the present study, using two durum wheat cultivars, the effect of increasing Cd supply on the development of Cd toxicity symptoms on leaves, and root and shoot growth, and concentration of Cd, ascorbic acid and non-protein SH-groups in roots and shoots was studied.

Materials and Methods

Plant Growth

Seeds of two cultivars of *Triticum durum* (Balcali-85 and C-1252) were germinated in perlite for 5 days. The seedlings were then transferred to plastic vessels (24 seedlings per vessel) containing aerated nutrient solution. The nutrient solution consisted of 0.88 mM K_2SO_4 , 2.0 mM $Ca(NO_3)_2$, 0.25 mM KH_2PO_4 , 1 mM $MgSO_4$, 0.1 mM KCl, 100 μ M Fe-EDTA, 1 μ M H_3BO_3 , 0.5 μ M $MnSO_4$, 1 μ M $ZnSO_4$, 0.2 μ M $CuSO_4$ and 0.02 μ M $(NH_4)_6Mo_7O_{24}$. The water used for preparing the nutrient solution was deionized.

Plants were grown in a growth chamber under controlled environmental conditions (light/dark regimes of 16/8 h, temperature 22/18 °C, and humidity 55/65% and photosynthetic photon flux 350 μ mol $m^{-2} s^{-1}$ at plant height). After plants were grown for 4 days in nutrient solution, they were supplied with increasing concentrations of Cd (0, 6, 30, 75 and 150 μ M) in the form of $CdSO_4$ for 2 weeks. Nutrient solutions were changed every 3-4 days. The plants were harvested when the effects of increasing Cd supply on their growth became severe (e.g., after 14 days of growth with Cd supply under given conditions). Before harvesting, plants were assessed in terms of the severity of Cd toxicity on the leaves, and the roots were washed in $CaSO_4$ for 15 min and then in deionized water to remove Cd adhered to the roots' surface and within the roots' apoplasmic spaces. At harvest, the roots and shoots were separated and dried at 70 °C in order to determine dry weight and Cd concentration.

For measuring ascorbic acid and non-protein SH groups in fresh tissues, samples were then from the roots and whole shoots and stored in liquid nitrogen until analysis.

Cadmium Analysis

Dried and ground plant samples were digested at 500 °C and the ash was dissolved in 3.3% (v/v) HCl. Cd in digested samples was measured by inductively coupled argon plasma emission spectrometry (Jobin Yvon-Ultrace 138, France). The Cd measurement was checked against certified Cd values in reference plant samples obtained from the National Institute of Standards and Technology (Gaithersburg, USA).

Analysis of Ascorbic Acid and SH-Groups

The measurement of total ascorbic acid and non-protein SH groups was carried out as described in Cakmak and Marschner (1992). Approximately 0.5 g fresh leaf and 1.0 g fresh root samples were extracted with 5 ml of 5% *meta*-phosphoric acid, and centrifuged at 15,000 g for 15 min. For the assay of SH groups, the reaction mixture contained 0.5 ml aliquot of the supernatant, 2.5 ml 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA and 0.5 ml 6 mM 5-5'-dithiobis-(2-nitro-benzoic acid). Following incubation at room temperature, the color produced was measured at 412 nm with a spectrophotometer (Hitachi U-2000, Japan). Reduced glutathione (GSH) was used as a standard in the range 0 to 100 µg ml⁻¹. The total amount of ascorbic acid was measured after the reduction of oxidized ascorbic acid (dehydro ascorbic acid) to reduced form using DTT (1,4 dithiothreitol). The reaction mixture contained 0.2 ml aliquot, 0.5 ml 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, 0.1 ml 10 mM DTT and 0.1 ml 0.5% (w/v) *N*-ethylmaleimide (NEM) to remove excess DTT. In the reaction mixture, the color was developed after the addition of the following reagents: 0.4 ml 10% trichloroacetic acid (TCA), 0.4 ml 44% *ortho*-phosphoric acid, 0.4 ml 2,1'-bipyridine in 70% ethyl alcohol and 0.2 ml 3% FeCl₃. The mixtures were then incubated in a water bath at 40 °C for 40 min and the color produced was read at 525 nm. *L*(+)ascorbic acid was used as a standard in the range 0 to 100 µg ml⁻¹.

Treatments were randomized throughout the growth chamber and the results given in the tables and figures are the means from at least three independent replications. The results were statistically analyzed by Duncan's test at a 5% probability level.

Results

An increasing supply of Cd resulted in significant decreases in the shoot and root growth of both cultivars (Figs. 1 and 2). The decreases were more distinct in C-1252. For example, with a 6 µM Cd supply, shoot dry weight was reduced by around 20% in Balcali-85 and 40% in C-1252. Similar decreases were also noted for root dry weight. When compared to Balcali-85, C-1252 also showed more severe Cd toxicity symptoms on the leaves (Fig. 1). The typical symptom was the expression of necrotic patches on the bases and sheaths of the oldest leaves. Over time, the middle-aged leaves also became necrotic and the oldest leaves totally collapsed. C-1252 appeared to have a higher susceptibility to Cd toxicity than Balcali-85. It was interesting to note that the decreases in growth caused by Cd supply in C-1252 were no longer significant following 6 µM Cd supply (Fig. 2).

As expected, increasing Cd supply markedly enhanced the Cd concentration of plants (Table 1). C-1252 tended

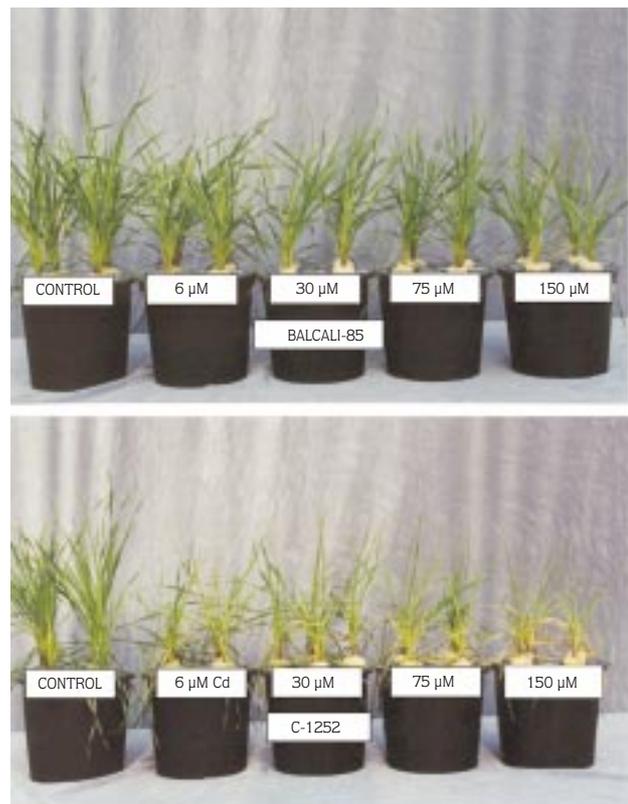


Figure 1. Growth of durum wheat cultivars Balcali-85 and C-1252 as affected by increasing Cd supply in nutrient solution for 18 days.

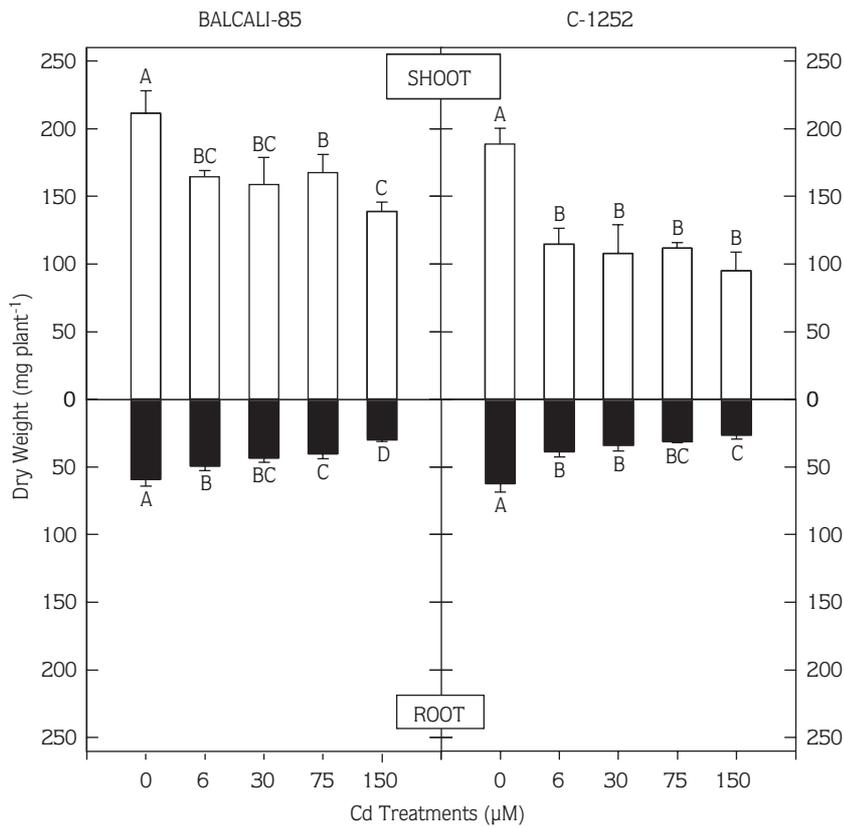


Figure 2. Effect of increasing Cd supply on shoot and root dry weight of durum wheat cultivars (Balcali-85 and C-1252) grown in nutrient solution for 18 days. The data represent means ± SD of three independent replications. Values carrying different letters are significantly different at P < 0.05.

Table 1. Effect of increasing Cd supply on shoot and root Cd concentrations of durum wheat cultivars (Balcali-85 and C-1252) grown in nutrient solution for 18 days. The data represent means ± SD of three independent replications.

Cultivar	Cd supply (μM)	Cd concentration (μg g ⁻¹ DW)	
		Shoot	Root
BALCALI-85	0	N.D.*	N.D.
	6	67 ± 2	1518 ± 172
	30	80 ± 5	3086 ± 419
	75	124 ± 11	3962 ± 234
	150	272 ± 44	4921 ± 743
LSD (5%)		40	789
C-1252	0	N.D.*	N.D.
	6	85 ± 5	1290 ± 47
	30	98 ± 3	2158 ± 242
	75	156 ± 12	2746 ± 78
	150	310 ± 33	33 ± 145
LSD (5%)		29	248

* could not be determined

to contain a higher Cd concentration in the shoots, but less in the roots, indicating that Balcali-85 has the better ability to retain Cd in the roots. Table 1 also shows that irrespective of cultivar, Cd was accumulated in the roots in much higher amounts than in the shoots, especially in the case of Balcali-85. For example, with 30 μM Cd, the Cd concentration was about 40-fold and 22-fold higher in the roots than in the shoots of Balcali-85 and C-1252, respectively.

As shown in Figure 3, ascorbic acid concentrations were significantly decreased in the shoots by Cd supply. These decreases were very similar in the two cultivars, and not pronounced with further increases in Cd supply up to 150 μM . In the case of the roots, the ascorbic acid concentration was first increased more than two fold in both cultivars when Cd was supplied at 6 μM (Fig. 3). However, at Cd supplies higher than 30 μM , the ascorbic

acid concentration showed a clear decrease in the roots, especially in C-1252. In addition, in the case of root ascorbic acid concentrations, the two cultivars did not clearly differ.

Increasing Cd supply caused a slight increase in non-protein SH groups in the shoots of both cultivars (Fig. 4). Interestingly, when Cd was not supplied, shoot concentrations of SH groups were about four fold higher than those in the roots. However, in the case of increasing Cd supply, SH groups showed massive increases in the roots, especially in Balcali-85. For example, when the Cd supply was raised from 0 to 30 μM , the root concentration of SH groups increased 30-fold in Balcali-85 and 18-fold in C-1252. In the control plants (no Cd treatment), the shoots had greater amounts of SH groups, while Cd-supplied plants contained more SH groups in the roots (Fig. 4).

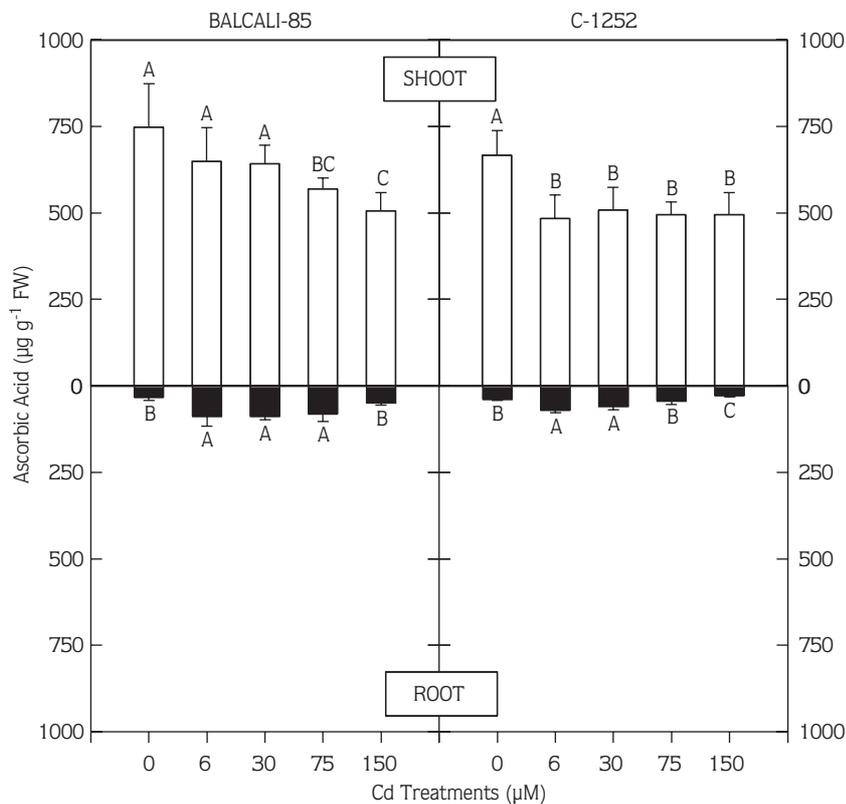


Figure 3. Effect of increasing Cd supply on shoot and root ascorbic acid concentrations of durum wheat cultivars (Balcali-85 and C-1252) grown in nutrient solution for 18 days. The data represent means \pm SD of four independent replications. Values carrying different letters are significantly different at $P < 0.05$.

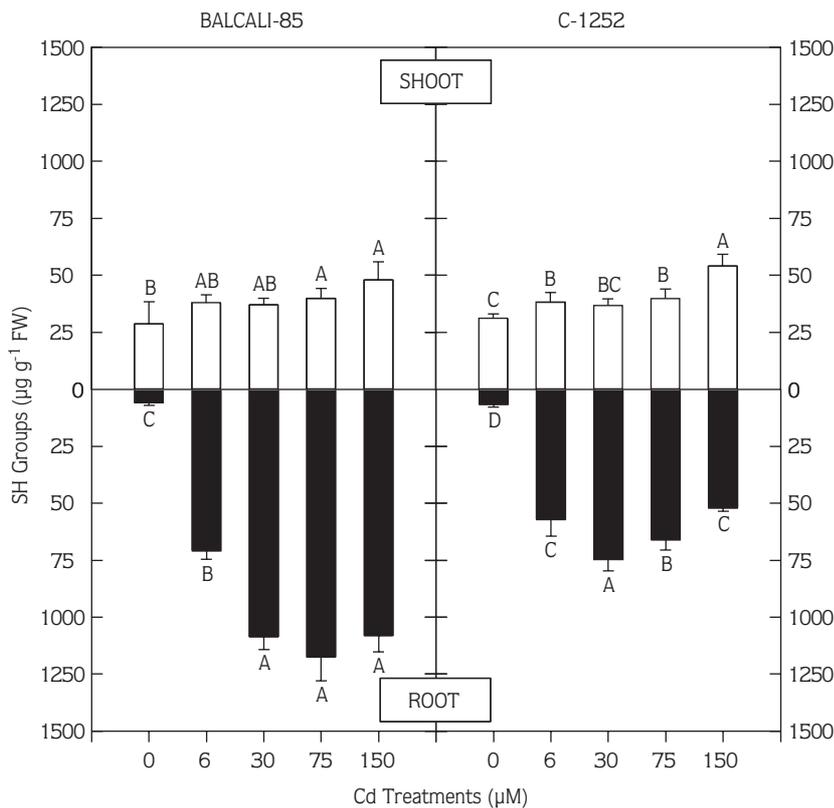


Figure 4. Effect of increasing Cd supply on concentrations of non-protein SH groups in roots and shoots of durum wheat cultivars (Balcali-85 and C-1252) grown in nutrient solution for 18 days. The data represent means \pm SD of four independent replications. Values carrying different letters are significantly different at $P < 0.05$.

Discussion

When compared to Balcali-85, C-1252 showed higher sensitivity to Cd toxicity in terms of the symptoms observed on its leaves. By increasing Cd supply, C-1252 showed more severe and earlier leaf symptoms of Cd toxicity and inhibition of shoot elongation. This higher sensitivity was associated with corresponding decreases in shoot growth (Figs. 1 and 2). In view of these observations, C-1252 can be classified as a Cd-sensitive cultivar.

Irrespective of cultivar, Cd supply reduced shoot and root dry matter production to a similar extent (Fig. 2). Generally, the results of previous studies show that Cd affects root growth more severely than shoot growth (Ouzounidou et al., 1997; Vitoria et al., 2001). There are, however, contrasting reports showing that the effect of Cd on shoot and root growth is similar (Stolt et al., 2003), as was also the case in our study. It seems likely

that the effect of Cd on shoot and root growth varies depending on the experimental conditions (solution culture, Cd treatments, plant species etc.).

Genotypical variations in tolerance to Cd toxicity are well documented in the literature (Grant et al., 1998). However, the physiological mechanisms conferring tolerance to Cd toxicity are still not well understood. Differences in root uptake and shoot accumulation of Cd can be an important factor in explaining genotypical variations in tolerance to Cd toxicity (Kochian et al., 2002; Hall, 2002). The results in Table 1 show that Balcali-85 and C-1252 are not greatly different in terms of shoot concentrations of Cd. C-1252 tended to have more Cd in the shoots, but this small difference cannot be an explanation for its higher sensitivity to Cd toxicity. In contrast to the shoots, Balcali-85 had a higher Cd concentration in the roots than C-1252, an indication that Balcali-85 has a better genetic ability to retain Cd in the

roots, possibly by binding and sequestering in the vacuole (see below), which may contribute to higher Cd tolerance in this cultivar.

Another major mechanism affecting the expression of high Cd tolerance is related to the antioxidative defense capacity of plants (Dixit et al., 2001; Hegedüs et al., 2001; Balestrasse et al., 2001). To understand the contribution of the antioxidative defence system of both cultivars to differential expression of Cd tolerance, we examined ascorbic acid and glutathione (non-protein SH-groups), which are major antioxidants in plant cells (Foyer and Noctor, 2000; Conklin, 2001). Both antioxidants are involved in the detoxification of reactive O₂ species in plants. The presented results showed that the durum wheat cultivars do not differ in ascorbic acid concentrations either in the roots or in the shoots, leading to the suggestion that ascorbic acid is not involved in the differential expression of Cd tolerance between Balcali-85 and C-1252.

Most of the non-protein SH groups in plants represent glutathione (Grill et al., 1979). Glutathione is involved not only in the detoxification of ROS, but is also essential for the synthesis of Cd-binding peptides such as phytochelatins, which inactivate and sequester Cd by forming stable Cd-complexes in the vacuole (Cobbett, 2000; Hall, 2002). The concentration of SH groups (glutathione) was less affected in the shoots, but was

strongly increased in the roots by Cd supply (Fig. 3). The increases in the SH-groups of roots by Cd were more marked in Balcali-85 than in the Cd-sensitive C-1252 (Fig. 4). For example, at the highest Cd supply, roots of Balcali-85 contained two fold more SH groups than the roots of C-1252. This indicates that SH groups are possibly involved in the greater Cd tolerance of Balcali-85 either by increasing the antioxidative defense mechanism or enhancing Cd-binding peptides. Greater increases in the SH groups of roots can enhance the production of Cd-binding proteins and thus the formation of Cd complexes in roots. Consequently, Cd taken up by roots cannot be transported into the shoots or sequestered in vacuoles. The importance of Cd-binding proteins in the development of Cd tolerance in plants has been shown by several studies (Howden et al., 1995; Clemens, 2001; Hall, 2002); these possibilities need to be investigated in Balcali-85 and C-1252 in future studies.

In conclusion, the results presented show the existence of genotypical variations in the tolerance to Cd toxicity among durum wheat cultivars. The differential tolerance to Cd toxicity in durum wheat was not related to ascorbic acid concentrations in the roots or shoots. In contrast to ascorbic acid, non-protein SH groups (glutathione) and the retention of Cd in the roots appeared to be related to differential Cd tolerance in durum wheat.

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