# CADMIUM UPTAKE AND ANTIOXIDATIVE ENZYMES IN DURUM WHEAT CULTIVARS IN RESPONSE TO INCREASING Cd APPLICATION

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## ABSTRACT

Effects of increasing cadmium (Cd) application rates on shoot and root growth, uptake and accumulation of Cd, and activity of antioxidative defense enzymes were studied in two durum wheat (*Triticum durum*) cultivars (cvs. Balcalı-2000 and Balcalı-85) differing in Cd tolerance. These durum wheat cultivars have been selected from a screening study including 10 bread and 6 durum wheat cultivars. The selected cultivars were grown in nutrient solution under controlled environmental conditions and subjected to increasing Cd concentrations (e.g., 0, 0.5, 2, 10, and 30 μM Cd). Genotypic variation in tolerance to increasing Cd stress was observed based on the development of necrotic patches on the base of the oldest leaves and reduction in dry matter production. Based on these parameters Balcalı-85 was ranked as the Cd-tolerant and Balcalı-2000 the Cd-sensitive genotype. The results of the root uptake and accumulation of Cd in root and shoot showed that the distinct genotypic difference in tolerance to Cd toxicity between two durum wheat cultivars was very closely related to the differential partitioning of Cd between roots and shoots. Both cultivars responded in a very similar way in total uptake of Cd by roots, but differed greatly in root accumulation and root-to-shoot transport of Cd. Compared to Balcalı-2000, Balcalı-85 had higher capacity to retain Cd in roots and reduce Cd transport into shoots. Consequently, in Balcalı-2000 the shoot concentration and content of Cd were nearly 2-fold higher than that of Balcalı-85, indicating a possible detoxification mechanism existing in Balcalı 85 to retain of Cd in roots and prevent photosynthetic tissues from Cd toxicity. Genotypic variation was also studied in terms of antioxidative enzymes including ascorbate peroxidase (AP), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT). The results obtained did not show any consistent relationship between Cd tolerance and activities of the antioxidative enzymes. At the highest level of Cd applications there was a clear decrease in $O_2^{\cdot-}$(superoxide)-generating NADPH oxidase activity in both cultivars, possibly due to inactivation of the enzyme by binding of Cd to NADPH.

The results obtained in the study clearly demonstrated that differential tolerance to Cd toxicity between durum wheat cultivars is related very closely to detoxification mechanism of the absorbed Cd in roots (e.g. binding to cell wall and/or compartmentalization in vacuole).



**Key words:** Cadmium, durum wheat, Cd uptake, Cd accumulation, Cd detoxification, antioxidative enzymes.

# ÖZET

Farklı dozlarda Kadmiyum (Cd) uygulamalarının, yeşil aksam ve kök büyümesi, bitkide Cd alınımı ve birikimi, ve antioksidatif savunma enzimleri üzerindeki etkileri, Cd toleransı bakımından farklılık gösteren iki makarnalık buğday çeşidi (Balcalı-2000 ve Balcalı-85) kullanılarak araştırılmıştır. Sözü edilen çeşitler, 10 ekmeklik ve 6 makarnalık buğday çeşidi arasından ön tarama çalışması sonucunda belirlenmiştir. Seçilen çeşitler besin çözeltisi ortamında yetiştirilerek artan dozlarda Cd (0, 0.5, 2, 10, ve 30 μM Cd) uygulanmıştır. Artan Cd stresine duyarlılıktaki farklar yaşlı yaprakların gövdeye yakın kısmında nekrotik lekelerin oluşumu ve kuru madde ağırlıklarındaki azalmalar şeklinde gözlemlendi. Bu sonuçlara bağlı olarak Balcalı-85 Cd' a dayanıklı, Balcalı-2000 ise Cd' a duyarlı çeşit olarak sınıflandırıldı. Kadmiyum alımı ve bitkiye alınan Cd' un kök ve yeşil aksamdaki birikimi ile ilgili sonuçlar, çeşitler arasında Cd toksisitesine dayanıklılıkta görülen genotipsel farkın, Cd' un kök ve yeşil aksamda farklı dağılımıyla çok yakın bir şekilde ilişkili olduğunu göstermiştir. Bitkiye alınan toplam Cd' un miktarı çeşitler arasında önemli farklılık göstermezken Cd' un kökte birikimi ve köklerden yeşil aksama taşınmasında büyük farklar belirlenmiştir. Balcalı-85' in Cd' u köklerde tutma özelliğinden dolayı bu çeşitte yeşil aksama Cd taşınmasının az olduğu; buna karşılık Balcalı-2000'de ise yeşil aksam Cd konsantrasyonu ve birikiminin Balcalı 85'ten yaklaşık 2 kat daha fazla olduğu belirlenmiştir. Bu sonuçlar Balcalı 85' in köklerinde Cd' u tutarak bir detoksifikasyon mekanizması geliştirdiğini ve bu şekilde fotosentetik dokuların Cd toksisitesinden korunduğunu göstermektedir. Kadmiyum toksisitesindeki genotipsel varyasyon antioksidatif enzim aktiviteleri (askorbat peroksidaz, glutatyon redüktaz, süperoksit dismutaz ve katalaz) bakımından da araştırılmıştır. Elde edilen sonuçlar Cd toleransı ve enzim aktiviteleri arasında anlamlı bir ilşkinin olmadığını göstermiştir. Yüksek dozda Cd uygulamasında, superoksit radikalinin üretiminde rol alan NADPH oksidaz enzim aktivitesinde azalma saptanmıştır. Bu azalmanın nedeni Cd'un NADPH molekülüne bağlanması ve buna bağlı olarak enzimin inaktivasyonu şeklinde yorumlanmıştır.

Bu çalışmada elde edilen sonuçlar, makarnalık buğday çeşitleri arsındaki Cd' a karşı farklı toleransın, bitkiye alınan Cd' un köklerde (örneğin hücre duvarına bağlanarak ve/veya vakuoler kompartmentasyona uğrayarak) detoksifikasyona uğraması ile yakından ilgili olduğunu göstermiştir.

**Anahtar kelimeler:** Cd, makarnalık buğday, Cd alınımı, Cd taşınımı, Cd detoksifikasyonu, antioksidatif enzimler.

*To my father, mother and sister…*

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## TABLE OF CONTENTS

## ABREVIATIONS

AP: Ascorbate peroxidase

Asc: Ascorbate

CAT: Catalase

Cd: Cadmium

Cu: Copper

Cys: Cysteine

DAR: Dehydroascorbate reductase

DHA: Dehydroascorbate

DTT: Dithiothreitol

DW: Dry weight

GR: Glutathione reductase

GSH: Reduced glutathione

GSSG: Oxidised glutathione

FW: Fresh weight

$H_2O_2$: Hydrogen peroxide

HSPs: Heat shock proteins

ICP-OES: Inductively coupled plasma optical emission spectroscopy

MDA: Monodehydroascorbate

MDAR: Monodehydroascorbate reductase

MT: Metallothioneins

µg: Microgram

mg: Milligram

NADPH: Nicotinamide adenine dinucleotide

NBT: Nitro blue tetrazolium chloride

$^1O_2$ : Singlet oxygen

$O_2^{\cdot -}$ : Superoxide anion radical

$OH^{\cdot}$ : Hydroxyl radical

PC: Phytochelatin

PMSF: Phenylmethylsulfonyl fluoride

POD: Peroxidase

ROS: Reactive oxygen species

SOD: Superoxide dismutase

Zn: Zinc

## LIST OF FIGURES

## LIST OF TABLES

# 1 INTRODUCTION

Heavy metals are consequential pollutants that their discharge to the environment is transported between air, water and soils continuously. Soil metal contamination has come about since pre-historic times. As a result of industrialization, the level and the rate of contamination has broadened since last century. Cadmium (Cd) is a non-essential heavy metal that is highly toxic even at very low concentrations. Sources that cause Cd contamination of soils are both natural (e.g. soil parent material or naturally transported soil material rich in Cd) and anthropogenic (e.g. sewage sludge, aerial deposition, agricultural and mining waste disposal, manure and phosphate fertilizer application to agricultural soils) (Jung MC et.al, 1997; McLaughlin et al., 1999; Garret et al., 2000; McLaughlin et al., 2005). Such anthropogenic sources outweigh natural sources ten-fold. Exposure to Cd can cause a number of adverse effects on biological responses in animals and humans (Nakarida et al., 2003; Jelovcan et. al., 2003).

Accumulation of heavy metals in agricultural soils gained great consideration due to food safety issues and potential health problems. The main route of absorption of Cd for humans is via food chain. Via the food chain, Cd is accumulated in the plant leaves and in the liver and kidneys of animals and fish tissues. The World Health Organization (WHO) has demonstrated a provisional tolerable weekly intake (PTWI) for Cd at 7 μg/kg of body weight which corresponds to a daily tolerable intake level of 70 μg of Cd for the average 70-kg man and 60 μg of Cd per day for the average 60-kg woman. As indicated the kidney is the most critical target organ in human beings (Foulkes 1993). Tubular renal effects occurred at lower cadmium levels than previously demonstrated, and more important, glomerular effects were also observed (Akesson et

al., 2005). Cadmium is also known to produce harmful health effects on the livers and bones (Jarup et al., 2004; Urani et al., 2005). Most of the available epidemiological information on Cd has been obtained from Japanese populations in highly contaminated areas (Nishijo et al., 2004).

The limit of total Cd concentration in cultivated areas has been reported to be 3 mg $kg^{-1}$ soil, while this limit was around 0.1 mg $kg^{-1}$ soil for uncultivated areas (Alloway, 1995). Cadmium concentrations are considerably higher, reported as between 100 to 600 mg $kg^{-1}$, in the areas subjected to Cd exposure by mining (Ernst and Neilssen, 2000; Lombi et al., 2000), or dispersal of sewage sludge. It has been shown that after sewage sludge application, Cd solubility and crop uptake rate have increased, and this effect endured during a 10-year period (Hyun et.al., 1998). In another study, it has been reported that accumulation of Cd by application of sewage sludge occurs in the top soil, stating the remaining time of Cd in the top soil as 1000 years (Lombi et. al., 2000).

Heavy metal contamination of agricultural soils is a growing concern which causes metal uptake by food crops. For international trade, presence of toxic metals in agricultural products may have important implications (Zarcinas et.al., 2004a,b). Although Cd is a non-essential trace metal, when reached to high levels in agricultural soils, it can be easily absorbed by plants. Accumulation of Cd in food crops including, cereals, potatoes, vegetables and fruits is widely being investigated (Grant et. al., 1998). Root uptake of Cd from soil depends on the Cd concentration in the soil, the soil pH, level of organic matter and Zn concentration in the soil (Eriksson et.al., 1996; Ciecko et. al., 2001; Yu et. al., 2005). Not only these soil factors mainly affect chemical availablity of soil Cd to plant roots, but also the plant itself regulates the uptake (Oliver et. al., 1995; Wenzel., 1996). Most of the Cd absorbed is accumulated in the roots and very little amount of Cd is transported from roots into shoots. Retention of Cd in roots is advantageous, since entry of Cd into human food chain is reduced. According to Grant et. al. (1998) Cd concentration in grains reduced when Cd translocation from roots to shoots is restricted.

There are many visible symptoms occurring in plants due to Cd toxicity including; leaf roll, chlorosis, growth inhibition of roots and shoots, and eventually Cd

toxicity results in plant death. One of the most sensitive responses of plants to Cd toxicity is defined as the reduction of root elongation (Guo and Marschner, 1995). Before any of these visible symptoms arise, Cd can either decrease or increase the activity of several enzymes, including antioxidative enzymes. It is well established by many studies that Cd causes increased production of reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide ($H_2O_2$), thus resulting in induction of antioxidant responses (Sandalio et. al., 2001; Iannelli et. al., 2002; Milone et. al., 2003; Romero-Puertas et. al., 2004). It is believed that most of the cell damage caused by Cd toxicity in plants is catalyzed by ROS. Against the adverse effects of these reactive molecules, plants developed an effective defence mechanism including antioxidant molecules and enzymes. Among antioxidative enzymes, while superoxide dismutase (SOD) catalyses the detoxification of $O_2^{\cdot-}$, catalase, ascorbate peroxidase and gluatathione reductase, involve in detoxification of $H_2O_2$ (Asada, 1992; Foyer et. al., 1994).

Reactions of plant species greatly differ in response to Cd toxicity. This variation occurs not only among plant species but also genotypes of a given species (Grant et. al., 1998; Zhang et. al., 2002; Liu et.al., 2003; Greger and Löfstedt, 2004). In order to minimize the adverse effects of heavy metal exposure, plants develop a variety of metal tolerance mechanisms. Immobilization of Cd at the cell wall level in roots is the first important barrier against Cd stress (Nishizono et.al., 1989). Exclusion of Cd which prevents Cd ions entering the cytosol, synthesis of Cd complexing peptides or proteins such as phytochelatins and metallothioneins, vacuolar compartmentalization of Cd, and increase in antioxidative defense systems are the most critical factors involved in Cd detoxification in plants (Gekeler et. al., 1989; Neumann et. al., 1994; Sanita` di Toppi and Gabbrielli, 1999).

As mentioned, response to Cd toxicity greatly differs among plant species and also among cultivars within a given species. Like any other crop species, root uptake, root-to-shoot translocation of Cd and partitioning of Cd between plant organs can vary in a variety of plant species including wheat (Grant et. al., 1998). Most of the mechanisms behind these processes are still unknown (Grant et. al., 1998).

The goal of this study was to investigate different mechanisms involved in differential expression of Cd tolerance by using two durum wheat cultivars; Balcalı-2000 (Cd-sensitive) and Balcalı-85 (Cd-tolerant). As wheat is the most important source of caloric intake among all food crops in Turkey and many other countries, two durum wheat cultivars were used to collect information on uptake and accumulation of Cd. In addition, durum wheat cultivars generally accumulate more Cd than bread wheat cultivars (Greger and Löfstedt, 2004). Any difference in uptake and transport of Cd between two durum wheat cultivars can contribute to better understanding of why durum wheats accumulate more Cd than bread wheats. The effects of Cd stress on two contrasting durum wheat cultivars in Cd tolerance was also studied for the possible involvement of antioxidative defense systems in genotypic variation for Cd tolerance.

# 2 OVERVIEW

## 2.1 Soil Contamination with Cadmium

Soil contamination by trace elements has become a worldwide concern since the levels of many heavy metals in some food crops have approached health limits in the past decades, such as Cd. Natural occurrence of Cd can be a result of volcanic emissions and Cd is also naturally originated from parent materials of soils. One of the natural sources of Cd is rock phosphates. Cadmium content of rock phosphate varies due to its geographical origin. The average concentration of Cd in worldwide soils is approximately 0.06 mg $kg^{-1}$ and it is 20-800 mg $kg^{-1}$ in metal-rich soils due to metal contamination or particular parent materials (He et. al., 2005). Main factors that cause significant contamination of soils with Cd are anthropogenic (Robards and Worsfold, 1991). Primarily, industrial activities, mining operations and applications of Cd-contaminated fertilizers (such as, phosphate fertilizers) are the main reasons of contamination which have been shown by many studies. In a recent study it has been demonstrated that with increasing distance from mine, metal concentrations decreased, indicating the main cause of contamination as mining (Tembo et. al., 2005). In another study, significantly higher values in the mean Cd concentrations in soil, plant, and human blood samples collected near a cement factory have been reported, showing importance of industrial activities in contamination of environment with Cd (Isıklı et. al., 2005).

The major sources of contamination in agricultural soils are the sludge-based fertilizers and phosphate fertilizers (Robards and Worsfold, 1991). On farmland

sewage sludge applications represent an important source of several trace elements including Cd. After incorporation of the sludge into soil, Cd becomes bio-available and easily mobilized. It has been shown that throughout a 10-year period after termination of sewage sludge application, Cd solubility and crop uptake increased (Bergkvist et. al., 2003). Chaudri et. al. (2001), demonstrated that following sludge application, the relationship between soluble Cd and grain Cd concentrations was more linear than it was between soluble Cd and total soil Cd concentrations; indicating that, even at low soil exposure levels, some crops may take up and accumulate greater concentrations of Cd into their edible parts.

The major processes that regulate the mobility and availability of Cd and other trace elements in the soil solution include; precipitation-dissolution, adsorption-desorption, ion exchange, soil and solution phase composition. Soil chemical properties such as pH, organic matter content, charge characteristics like ion exchange capacity greatly influence activity of Cd and thus uptake of Cd by plant systems (Kookana and Naidu, 1998; He et. al., 2005). Higher solubility of Cd in soil is the most critical cause of higher Cd concentration and uptake by plants (Grant et. al., 1998; Yanai et. al., 2006). Soil pH greatly affects chemical availability of Cd by altering its chemical form and mobility to plant roots. Commonly there is an inverse relationship between soil pH and Cd uptake by roots considering that Cd concentration of plant tissue decreases with increased soil pH when all the other properties of soil are remained constant (Singh and Myhr, 1998; Adams et. al., 2004; Yanai et. al., 2006). Another soil factor which has also effects on the availability of Cd to plant roots is the cation exchange capacity of soils. There is also interaction between metals during their uptake and transport in plants. As expected, soils with high content of clay minerals have more Cd adsorption and fixation capacity than other soil types (Grant et. al., 1998). Interaction of Cd and Zinc (Zn) plays a significant role in this process. In most cases, antagonistic interactions of the two metals are found in Cd and Zn uptake by plant roots (Cakmak et. al., 2000; Koleli et. al., 2004; Salah and Barrington, 2005).

## 2.2 Cadmium in Plant Systems

### 2.2.1 Cellular Mechanisms in Response to Cadmium Stress

Plants develop a number of defense mechanisms at the cellular level in response to Cd stress. Most of these mechanisms are involved in the detoxification of Cd at cellular level. When Cd is absorbed at high levels, avoidance from building up toxic forms of Cd at receptive sites within the cell, is the main basis of the defense systems in living organisms. Upon exposure to high Cd, plant cells can accumulate huge amount of Cd-binding chelators or peptides which inactivate physiological activity of Cd in plants. These Cd-binding compounds have been discussed below, in detail.

Since the root cell wall is the first contact with metals in the soil solution, it acts as the first blockage against Cd stress via immobilization process. Depending on the Cd concentration and the species, metal specific tolerance by means of the cell wall can be different. It has been reported that in the roots and leaves of bush bean, Cd ions seem to be mostly bound by pectic sites and hystidyl groups of the cell wall (Leita et al., 1996). In another study it has been shown that in *Silene vulgaris* ssp. *humilis* metals that are accumulated in the epidermal cell walls are either bound to a protein or silicates (Bringezu et. al., 1999).

Presence of heavy metals in elevated concentrations in plant tissues may cause increased leakage from cells. Damage to the membrane by Cd may be due to various mechanisms including oxidation and cross-linking of protein thiols, inhibition of key membrane proteins such as the $H^+$-ATPase, or changes to the composition of membranes (Meharg, 1993). According to many reports (Sandalio et.al., 2001; Ali et. al., 2002; Ranieri et al., 2005; Smeets et. al., 2005) Cd toxicity represents an oxidative stress catalysed by highly toxic reactive oxygen species (ROS). In some plant genotypes high Cd tolerance is associated with high antioxidative defense mechanisms that will be discussed in detail below. Preventing the access of Cd ions into cytosol via activated efflux mechanisms through the action of plasma membrane is another important plant defense mechanism against Cd toxicity. Plants can also release some organic

compounds from roots into rhizosphere to complex Cd and inhibit its uptake (Marschner, 1995).

#### 2.2.1.1 Phytochelatins

Among heavy metal detoxification and tolerance mechanisms chelation of metals in the cytosol by high affinity ligands takes an important place. These ligands may be aminoacids and organic acids, and two most significant classes of peptides are phytochelatins (PC) and the metallothioneins (MT) (Hall, 2002).

A system related to sulphur metabolism is activated when Cd has entered the cytosol which causes production of Cd-complexing agents PCs. The general structure of PCs is $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ where *n* is the number of repetition of the unit γ-Glu-Cys varying between 2-11. Due to the presence of thiolic groups of cysteine (Cys) which chelate Cd, PCs form complexes with Cd that result in prevention of Cd circulating as free $Cd^{2+}$ inside the cytosol (Grill et. al., 1985). Cd-PC complex is shown to be up to 1000 times less toxic to many plant enzymes than the free Cd is (Kneer and Zenk, 1992). PCs are synthesized from glutathione as substrate by enzyme phytochelatin synthase (Grill et. al, 1989). Within a few minutes after Cd supply the enzyme is self-regulated and synthesized PCs to chelate Cd. Chelation of Cd is required to activate the enzyme and the reaction continues until Cd is not supplied (Loeffler et al., 1989).

In many studies the crucial role of PCs in detoxification and tolerance has been demonstrated. Cd-sensitive mutants of *Arabidopsis* that differed in their ability to accumulate PCs were isolated. There was a negative correlation between the amount of PC accumulated and the level of Cd sensitivity (Howden et.al., 1995a; b). In another study using *Arabidopsis* it has been shown that Cd and copper (Cu) treatment enhanced transcription of the genes for glutathione synthesis, and the response was metal-specific (Xiang and Oliver, 1998). As shown in the cultured cells of azuki beans there was a close relationship between high Cd sensitivity and lack of phytochelatin synthase (Inouhe et. al., 2000). In a recent study, *Triticum aestivum* leaves exposed the Cd stress was counteracted by PC biosynthesis (Ranieri et. al., 2005).

#### 2.2.1.2 Vacuolar Compartmentalization

Transport of heavy metals like Cd into the vacuole is a phenomenon that is important for reducing the levels of heavy metals in the cytosol. Since vacuolar compartmentalization keeps Cd ions into a limited space, free circulation of the ions in the cytosol is prevented. This accumulation appears to be mediated by both a $Cd^{2+}/2H^{+}$ antiporter and an ATP dependent ABC transporter located at the tonoplast (Salt and Wagner, 1993; Salt and Rauser, 1995; Rea et. al., 1998). Vacuolar transport of PC-Cd complexes in the root cells might delay the radial Cd transport to the xylem and further transport to the shoot.

Synthesis of PCs takes place after exposure to Cd that results in the rapid formation of a low molecular weight (LMW) complex with Cd. Also a medium molecular weight (MMW) PC-Cd complex has been described with a higher polymerization level. These two complexes obtain acid-labile $S^{2-}$ at the tonoplast level in order to form a high molecular weight (HMW) complex which has a higher affinity towards Cd ions. Ability of PCs to link $S^{2-}$ groups quickly, in order to bind free Cd ions efficiently is critical for Cd detoxification. Therefore, the HMW complex highly stabilized by $S^{2-}$ groups plays a crucial role in detoxification process (Sanita` di Toppi and Gabbrielli, 1999; and the references therein) (Fig 2.1).

Since the pH is acidic in the vacuole, the HMW complex dissociates. As a result Cd can form new complexes with vacuolar organic acids such as; citrate, oxalate, malate and aminoacids. Vacuolar hydrolases may degrade apo-phytochelatins resulting in going back to the cytosol in order to carry out their shuttle role (Sanita` di Toppi and Gabbrielli, 1999; and the references therein) (Fig 2.1).

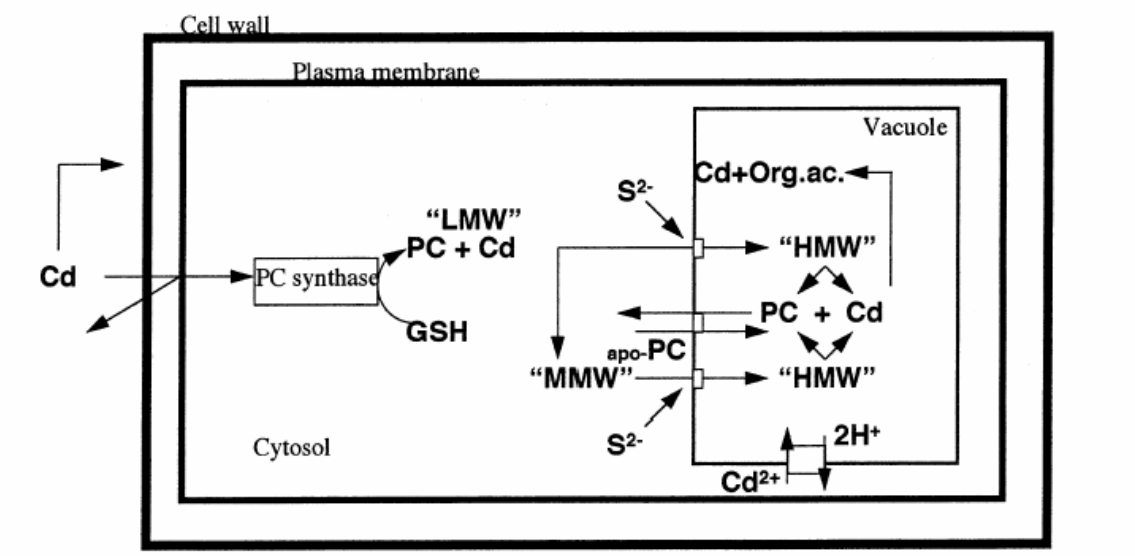


**Figure 2.1** Schematic representation of the mechanisms involved in Cd chelation and compartmentalization in the vacuole. 'LMW', low molecular weight complex; 'MMW', medium molecular weight complex; 'HMW', high molecular weight complex; GSH, glutathione; PC, phytochelatins; apo-PC, apo-phytochelatins; $S^{2-}$, acid-labile sulphur; Org. ac., organic acids (Sanita` di Toppi and Gabbrielli, 1999).

### 2.2.1.3 Metallothioneins

Metallothioneins (MTs) are the second type of cysteine-rich metal binding peptides generally lacking aromatic aminoacids in higher plants. Cys residues are present in plant MTs as Cys-x-Cys, Cys-x-x-Cys where x is an amino acid other than Cys or Cys-Cys clusters (Sanita` di Toppi and Gabbrielli, 1999). Class 1 MTs have Cys residues aligning with mammalian renal MT. Class 2 MTs can not be aligned with MT1, but have similar Cys clusters (Robinson et. al., 1993; Prasad, 1999). In a variety of plants in addition to MT1 and MT2 genes, MT3 and MT4 types have been distinguished (Hall, 2002). The majority of plant MT genes have been identified in the angiosperms. A number of species, including *Arabidopsis*, rice, and sugarcane contain genes encoding all four types of MTs (Cobbett and Goldsbrough, 2002).

More information is needed about the distribution and form of MTs and their metal-binding properties in plants. Even though it has been difficult to study MT proteins in plants, several plant MTs have been expressed in microbial hosts in order to test the metal-binding properties of these proteins and their ability to provide metal tolerance. It has been shown that the pea MT1, PsMTa, bound to Cu, Cd and Zn when expressed in

*E.coli*. Highest affinity was displayed for Cu (Tommey et. al., 1991). Induction of MT1 genes by a variety of stresses, including aluminum (Al), Cd, nutrient deprivation, and heat shock was also demonstrated in rice. Altough there was not a direct connection to metal ion status, MTs were expressed as a part of general response (Hsieh et. al., 1995).

In animals, MTs has a protection role against Cd toxicity but this function in plants is clearly provided by PCs (Cobbett and Goldsbrough, 2002).

#### 2.2.1.4 Stress Proteins

Synthesis of heat shock proteins (HSPs) is enhanced by an exposure to stress factors including heavy metal toxicity. Production of specific mRNA transcripts which controls the synthesis of stress proteins by Cd-stressed cells has been demonstrated by many studies (Sanita` di Toppi and Gabbrielli, 1999). In rice both heat and heavy metal stress increased the levels of mRNAs for low molecular mass HSPs (Tseng et. al., 1993). In cell cultures of *L. peruvianum* exposed to 1 mM Cd, HSP70 were bound to plasmalemma, mithocondrial membranes, and to the endoplasmic reticulum in noticible amounts (Neumann et. al., 1994). In the same study, it has been shown that a short heat stress applied before the heavy metal stress helped prevention of membrane damage caused by Cd. It has been reported that Cd induced the production of the stress proteins with a molecular mass of 42.000 in *Phaseolus vulgaris* (Leita et. al., 1991).

## 2.3 Cadmium Uptake and Translocation

Plants have ability to regulate Cd uptake by roots (Salt et. al., 1995). Uptake of Cd at the root surface has been characterized in many species comprising wheat, maize and barley (Grant et al., 1998; Hart et. al., 1998). As plants absorb water for transpiration, Cd is taken up from the soil solution. In most cases influx of Cd at the root plasma membrane level is due to a concentration-dependent process via a carrier mediated

system (Das et. al., 1997; Hart et. al., 1998). Cadmium taken up by plants is mostly accumulated in the roots.

Genotypic variation in Cd uptake has been shown among many plant species as well as within the cultivars of a given species (Li et. al., 1997; Grant et. al., 1998). Substantial variability among 99 pea genotypes in tolerance to Cd and uptake of different heavy metals was reported (Belimov et. al., 2003). In another study it has been demonstrated that lower level of seed Cd in certain soybean varieties was due to the lower initial uptake (Arao et. al., 2003).

In view of the fact that maintenance of Cd in the roots decreases its transport to other parts of the plant, translocation of Cd is an important determinant while exploring genotypic differences in Cd tolerance (Arao et. al., 2003). Rather than the root uptake, translocation seems to be the source of variation in differential shoot Cd concentration. Transport of metals from roots to shoots is a process driven by transpiration from leaves that includes metal uploading into root xylem cells, long distance transport from roots to shoots within xylem and reabsorption of metals from the xylem stream by leaf mesophyll cells (Raskin and Ensley, 2000). In a recent study, it has been demonstrated that differences in shoot Cd accumulation is unrelated to transpiration. In the absence of transpirational flow, the timing and magnitude of isoline differences in Cd concentration of root pressure xylem exudates closely followed the pattern of the root-to-shoot Cd translocation in intact plants (Harris and Taylor, 2004). In the same study it has been clearly shown that difference in Cd accumulation in shoots of a pair of near-isogenic durum wheat lines depends on the difference in the root-to-shoot Cd translocation. Cd translocation in the high grain-Cd isoline was 1.8 fold higher than the low grain-Cd isoline. There was no difference between isolines in Cd uptake by roots. In peanut plants significant cultivar differences were identified by means of Cd distribution in plants while there were no important variations between the cultivars in terms of total Cd uptake (McLaughlin et al., 2000). Greger and Löfstedt (2004) showed that the translocation among the low-Cd accumulators is likely to be the lowest for winter bread wheat, followed by spring bread wheat and the durum wheat. Moreover, translocation among the durum wheat cultivars appears to be higher, 350 to 450 $\mu g\ mg^{-1}$, when compared to bread wheat cultivars. When time dependent uptake and translocation of Cd is considered, plants may give different responses. It has been

demonstrated that in durum wheat plants short-term accumulation of Cd into roots began to decrease after 4-6 h exposure. During that period there were no differences between isolines. Differences in Cd accumulation in shoots were observed after 24 h of exposure (Archambault et. al., 2001).

## 2.4 Generation and Detoxification of ROS in Plants

In plant cells chloroplast, mitochondrial and plasma membrane-linked electron transport processes causes the leakage of electrons onto molecular oxygen, thus resulting in an unavoidable production of reactive oxygen species (ROS), such as the superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide ($H_2O_2$), singlet oxygen ($^1O_2$) and hydroxyl radicals ($OH^{\cdot}$) (Rubinstein and Luster, 1993; Asada, 1994; Fridovich, 1995). In the case of abiotic stresses such as heat, drought, extreme temperatures, UV radiation, mineral nutrient deficiency and heavy metals, production ROS is enhanced particularly in chloroplasts and mithocondria (Bowler et. al., 1992; Smirnoff, 1993; Foyer et. al., 1994; 1997). An imbalance in the regeneration and removal of ROS causes oxidative stress and related cell damage. Induction of oxidative stress by Cd occurs, probably, through indirect mechanisms such as interaction with the antioxidative defense, disruption of the electron transport chain or induction of lipid peroxidation and chlorophyll degradation (Somashekaraiah et. al., 1992; Chaoui et. al., 1997; Leon et. al., 2002; Chen et. al., 2003).

Due to the accumulation of ROS which leads to an oxidative burst, cellular damage is increased via oxidation of several critical macro-molecules such as lipids and proteins (Hall, 2002). These adverse effects of Cd cause inactivation of photosystem II and inhibition of photosynthetic enzymes leading to reduced $CO_2$ fixation and activation of molecular $O_2$ to ROS, mainly under high light intensities (Foyer et. al., 1994; Foyer et. al., 1997). Although Cd toxicity induces oxidative stress in different plant species (Dixit et. al., 2001; Schützendübel et. al., 2002), Cd ions are however, unable to catalyze Fenton-Haber-Weiss reactions, which generate the most reactive free radical $OH^{\cdot}$. Some metals such as $Fe^{2+}$ and $Cu^{+}$ induce oxidative cell damage as a consequence

to their auto-oxidation, through Fenton-type or Haber-Weiss reactions. This reaction has not been described for $Cd^{2+}$ in plants because it is not a redox metal (Schützendübel and Polle, 2002).

Plants have enzymatic and non-enzymatic antioxidative protective mechanisms to deal with ROS which consist of enzymes in addition to antioxidants. These antioxidative mechanisms may directly take place in the removal of ROS or in the generation of the reduced state antioxidative substrates. Reducing metabolites, such as ascorbate (vitamin C), glutathione and α-tocopherol (vitamin E) have central roles in enzyme-catalyzed detoxification reactions. They act either as substrates or they directly scavenge toxic radicals in non-enzymatic reactions (Asada and Takahashi, 1987; Foyer 1993; 1997). Among the antioxidative enzymes, superoxide dismutase (SOD), catalase (CAT), monodehydroascorbate reducetase (MDAR), peroxidases (POD), ascorbate peroxidase (AP), dehydroascorbate reductases and glutathione reductase (GR) are the major part of the antioxidative defense system of plants and involved in detoxification of ROS in various compartments in the cell (Asada, 1994).

In enzymatic defense mechanism, SOD catalyzes the dismutation of $O_2^{\cdot -}$ to $H_2O_2$ and $O_2$ that results in the maintenance of a low steady state concentration of superoxide radical. By this way, hydroxyl radical ($OH^{\cdot}$) formation by $O_2^{\cdot -}$ catalyzed Fenton-Haber-Weiss reaction is minimized (Bowler et. al. 1992; Scandalios, 1993; Alscher et. al., 2002). When plants are exposed to environmental stresses generation of $O_2^{\cdot -}$ in chloroplasts is stimulated. Under environmental stress conditions, photosynthetic $CO_2$ fixation is limited, therefore electron flow often is intensified to $O_2$ instead of $CO_2$, resulting in production of $O_2^{\cdot -}$ and $O_2^{\cdot -}$ – derived ROS such as $H_2O_2$ and $OH^{\cdot}$ (Foyer et. al., 1997; Asada, 1999). Hydrogen peroxide is broken down by catalase and peroxidases (Asada, 1992; Scandalios, 1994). Since catalase have low affiniy to $H_2O_2$ and high sensitivity to light induced inactivation and localized in peroxisomes, the protective effect of CAT against $H_2O_2$ is limited (Cakmak and Marschner, 1992; Foyer et. al., 1994). Besides its adverse effects on oxidation of SH-containing enzymes of the Calvin Cycle, and consequently inhibition of photosynthesis, $H_2O_2$ is also responsible for the production of potent oxidant $OH^{\cdot}$ via Fenton reaction.

An alternative and more efficient ascorbate-dependent $H_2O_2$ – scavenging mechanism in plant systems is the "ascorbate-glutathione pathway" (Foyer et. al., 1997; Asada, 1999). In this detoxification system, which exists in chloroplasts and cytosol, $H_2O_2$ is reduced to $H_2O$ by the activity of acsorbate peroxidase (AP). In this reaction ascorbic acid is used as an electron donor while monodehydroascorbate (MDA) and dehydroascorbate (DHA) are formed at the end of the reaction. The regeneration of reduced ascorbate (Asc) from MDA or DHA can be catalyzed either by NADH-dependent monodehydroascorbate reductase (MDAR), or by GSH dependent dehydroascorbate reductase (DAR) coupled with glutathione reductase (GR). With the contribution of DHA, glutathione (GSSG) that was oxidized during the regeneration of ascorbic acid is again converted to the reduced form (GSH) through the activity of GR in a NADPH-dependent reaction (Cakmak, 1994; Foyer et. al., 1994) (Fig 2.2). The importance of ascorbate-glutathione pathway in cytosol for detoxification of $H_2O_2$ produced in seeds during germination has also been demonstrated (Cakmak et. al., 1993). Many studies have demonstrated the importance of the antioxidative defense mechanisms against oxidative stress induced by different stress factors. Cuypers et. al., (2000; 2001) has well demonstrated the involvement of these defense systems under heavy metal stress. In a very recent study, difference in salt tolerance between rice cultivars has been explained by antioxidative responses (Demiral and Turkan, 2005). In another study increased protection provided by increased activity of antioxidant enzymes under temperature-stress has been demonstrated (Ali et. al, 2005).

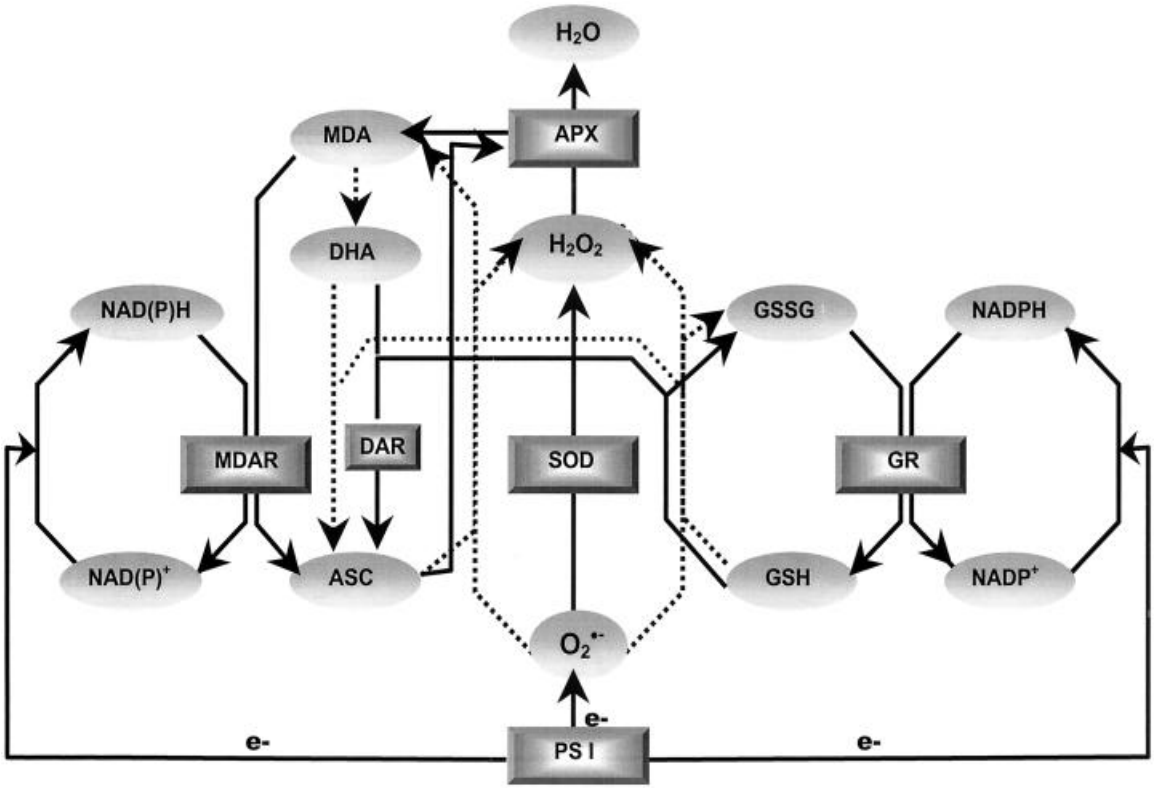


**Figure 2.2** The SOD-ASC-GSH cycle (bold lines, enzymatic reactions) and nonenzymatic redox reactions (dashed lines) (Polle, 2001). APX: Ascorbate peroxidase, ASC: Ascorbate, DAR: DHA reductase, DHA: Dehydroascorbate, GR: Glutathione reductase, GSH: Reduced glutathione, GSSG: Oxidised glutathione, MDA: Monodehydroascorbate, MDAR: MDA reductase, SOD: Superoxide dismutase.

Among the antioxidants that are involved in detoxification of ROS, ascorbate has a crucial role: It reacts directly with singlet oxygen and reduces superoxide ($O_2^{\cdot-}$) and hydroxyl radicals ($OH^{\cdot}$). Through the activity of AP, ascorbate indirectly eliminates $H_2O_2$ (Fig 2.2) (Foyer et. al. 1997; Noctor and Foyer, 1998). Ascorbate also acts as an electron donor in the regeneration of α-tocopherol. An additional important antioxidant is glutathione which involves in detoxification of singlet oxygen and $OH^{\cdot}$ , thus prevents SH-groups of enzymes from oxidation (Foyer et. al. 1997; Noctor and Foyer, 1998). Through the ascorbate-glutathione cycle GSH contributes to regeneration of ascorbate and α-tocopherol (Foyer 1994). A further role of glutathione in defense mechanisms of plants under heavy metal stress is its participation to synthesis of phytochelatins (PCs). PC accumulation has a central role in metal detoxification (Cobbett and Goldsbrough, 2002) and PCs are synthesized from reduced glutathione (GSH) which is non-protein thiol in plants (Cobett, 2000).

The effects of stress conditions on the production of ROS and the levels of enzymatic and non-enzymatic antioxidant defense mechanisms, especially in response to heavy metals, have widely been studied among different species (Cuypers et. al., 2000; 2001). Increasing and decreasing activity of many antioxidative enzymes have been observed. There are conflicting results in response to Cd induced oxidative stress both among species and genotypes within a species (Sandalio et.al., 2001; Ali et. al., 2002; Ranieri et al., 2005; Smeets et. al., 2005), and controversial reports in the literature regarding antioxidative defense enzyme activities under different Cd treatments as reviewed by Tiryakioglu et al. (2006).

Production of ROS can be induced when plants are exposed to various biotic and abiotic stresses. Induced production of $O_2^{\cdot-}$ is generally catalyzed by NADPH-oxidizing enzyme systems which are localized in cell walls, plasma membranes, cytosol and microsomes (Cakmak and Marschner, 1988; Cakmak 2000). Toxic $O_2^{\cdot-}$ species produced by NADPH-dependent oxidases are also involved in damage to several critical cell constituents. Structural and functional impairments in root cel membranes associated with the enhanced activity of $O_2^{\cdot-}$ -generating NADPH oxidase by different stress factors is demonstrated by different studies. Zn deficiency, Cu deficiency and toxicity were shown to be involved in the activation of this enzyme and $O_2^{\cdot-}$ production (Cakmak and Marschner, 1988; Quartacci et. al., 2001).The effect of Cd stress on this enzyme is poorly described. There is need for further studies investigating the role of Cd toxicity on NADPH-oxidases.

In this study, experiments were carried out under controlled environmental conditions to investigate differential Cd uptake, accumulation and antioxidative defense mechanisms in two wheat cultivars, Balcalı-2000 and Balcalı-85, differ in their response to Cd application. Also the role of Cd in superoxide-generating NADPH oxidase was studied.

# 3 MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 3.1.1 Plant Material and Growth Conditions

#### 3.1.1.1 Greenhouse Experiments

##### 3.1.1.1.1 Plant Material

To select the most tolerant and the most senisitive wheat genotypes, a screening experiment has been conducted by using 16 bread and durum wheat cultivars. A total of 10 bread wheat (*Triticum aestivum*, cvs. Aytin, Cetinel 2000, Yakar, Atay 85, AK702, Dagdas, Bolal, Bagci, ES 14 and 03KE12) and 6 durum wheat (*Triticum durum*, cvs. Selcuklu, Balcalı-2000, Zenit, Meram, Yilmaz and Balcalı-85) cultivars were used in the screening experiment carried out under greenhouse conditions. Seeds of the cultivars have been obtained from the Anatolian Agricultural Research Institute - Eskisehir and International Agricultural Institude – Konya.

##### 3.1.1.1.2 Growth Conditions

Plants were grown under greenhouse conditions in plastic pots containing 1700 g soil. The soil used was Zn deficient (0.1 mg Zn $kg^{-1}$ soil) and obtained from Central Anatolia. The main soil characteristics were: pH 8.04, $CaCO_3$ 14.9 %, organic matter

0.69 %, salt %0.08 and soil texture was clay (% 60.6). About 15 seeds were sown in each pot and after emergence the seedlings were thinned to 10 per pot at the two leaf stage. For the Cd treatment, Cd was applied at a rate of 20 mg $kg^{-1}$ soil in the form of $3CdSO_4.8H_2O$, together with a basal treatment of 200 mg N $kg^{-1}$ soil as $Ca(NO_3)_2$ and 100 mg P $kg^{-1}$ soil as $KH_2PO_4$, 125 mg K $kg^{-1}$ soil as $KH_2PO_4$, 20 mg S $kg^{-1}$ soil as $CaSO_4.2H_2O$, 2.5 mg Zn $kg^{-1}$ soil as $ZnSO_4.7H_2O$, 2.5 mg F $kg^{-1}$ soil as FeEDTA ($C_{10}$ $H_{12}$ $FeN_2$ $NaO_8$). Control pots were also treated with the basal nutrients excluding Cd. All nutrients were mixed thoroughly with soil before potting. The treatments were performed in triplicate and the pots were randomized every 4-5 days. Plants were watered daily with deionized water.

After 34 d of growth in the greenhouse, the shoots were harvested and dried at 70 ºC for determination of shoot dry matter production and Cd concentration in the whole shoot.

#### 3.1.1.2 Growth Chamber Experiments

##### 3.1.1.2.1 Plant Material

Based on the greenhouse screening experiment two cultivars of durum wheat, Balcalı-2000 and Balcalı-85 were selected and used in the experiments carried out under growth chamber conditions, as Cd-sensitive and Cd-tolerant genotypes respectively.

##### 3.1.1.2.2 Growth Conditions

Plants were grown under controlled environmental conditions (light/dark regime: 16/8 h at 20/18 ºC, relative humidity: 65-75 %, photon flux density: 700 µE $m^{-2}$ $s^{-1}$). Seeds were first sterilized with 1% (w/v) calcium hypochlorite for 10 min, and then sown in perlite moistened with saturated $CaSO_4$ solution and germinated in dark for 5 days in room temperature (22 ºC±2ºC). Afterwards, the seedlings were transferred to 2.5 L black plastic pots containing continuously aerated nutrient solution. The composition

of the nutrient solution was as follows: 2mM $Ca(NO_3)_2$, 1mM $MgSO_4.7H_2O$, 2 mM $K_2SO_4$, 0.2 mM $KH_2PO_4$, $10^{-6}$ M $H_3BO_3$, $10^{-6}$ M $MnSO_4.H_2O$, $10^{-6}$ M $ZnSO_4.7H_2O$, $2x10^{-7}$ M $CuSO_4.5H_2O$, $2x10^{-8}$ M $(NH_4)_6Mo_7O_{24}.4H_2O$, and $10^{-4}$ M FeEDTA ($C_{10}H_{12}FeN_2NaO_8$). After plants were grown for 5 days in nutrient solution, Cd was added to the nutrient solution at different concentration levels as indicated in the legend of relevant figures and tables. Cd was applied in the form of $3CdSO_4.8H_2O$.

After 7 days following Cd application plants were harvested. In most of the experiments plants at harvest were 17 days old. At harvest, roots and shoots were seperated. Roots were rinsed with 2 mM $CaCl_2$ for about 15 min to remove surface adsorbed Cd and then rinsed in deionised water throughly. Then, roots and shoots were dried at 70 °C for determination of dry matter production and Cd concentration. In the case of the Cd uptake experiment, plants were harvested after 3 days growth in the Cd supplied-nutrient solutions. During the Cd uptake experiment, nutrient solution has been sampled at different time intervals to measure depletion of Cd in nutrient solution. Measurement of antioxidant enzyme activities was carried out only on leaf samples. For the analysis of NADPH-dependent $O_2^{\cdot-}$ generation and NADPH oxidase, only roots were sampled. Harvested leaf and root samples were treated with liquid nitrogen and stored at -80°C until analysis.

## 3.2 Methods

### 3.2.1 Dry Matter Production and Cadmium Tolerance Index

Plants dried at 70°C were weighed for determination of dry matter production. The Cd tolerance index was calculated as the ratio of shoot (or root) dry weight at different Cd concentrations to that without Cd supply (control treatment) as following:

**Cd tolerance Index = (Dry Weight at Cd Supply/Dry Weight at Control Treatment) x 100**

### 3.2.2 Cadmium Concentration and Content

The dried root and shoot samples were then ground and approximately 0.2 g ground samples were ashed at 500 °C for 12 h for determination of Cd concentration. The ashed samples were dissolved in 3.3 % $HNO_3$ (v/v). The concentration of Cd was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian, Australia) at 214.439 nm emission wavelength. Cadmium measurement in plant materials were checked against certificated Cd values in different reference plant materials obtained from the National Institute of Standards and Technology (Gaithersburg,USA). The Cd content was calculated by multiplying the dry weight values of roots or shoots with their Cd concentration values. Translocation index of Cd from roots into shoots was calculated as the ratio of shoot Cd content to the total Cd content (shoot Cd content + root Cd content) as given below.

$$\text{Translocation Index} = \frac{\text{Shoot Cd Content}}{\text{Total Cd Content}} * 100$$

For the uptake experiments 10 ml nutrient solution samples from each pot were collected at different time intervals as indicated in the results section. As the water is absorbed by plants due to transpiration, before collecting sample solutions, each solution was filled to the initial volume to avoid any concentration effect due to reduced water level in pots. The concentration of Cd in the collected samples was measured by ICP-OES. The results were calculated in terms of root dry matter production, based on absorbtion of Cd in 1 hour in micromoles (μmol Cd $g^{-1}$ root DW $h^{-1}$) and calculated as cumulative Cd absorbtion based on absorbtion of Cd in micromoles (μmol Cd $g^{-1}$ root DW).

### 3.2.3 Determination of Soluble Protein Content

Protein content of shoots and roots was measured spectrophotometrically, using bovine serum albumin as a standard according to Bradford (1976). The protein assay reagent was prepared as follows: 100 mg coomassie brillant blue G 250 was dissolved in 50 ml absolute ethyl alcohol (99.5 %) and added with 100 ml of 85 % *ortho*-phosphoric acid. The mixture is filled up to 600 ml with deionised water and then filtered. Right after filtration 100 ml of glycerol (about 87 %) is added and filled up to 1000 ml with deionised water. Reagent was used after 24 hours in measurement of protein content of the enzyme extracts. For protein assay, 100 µl sample solution (root or shoot) and 5 ml assay reagent were mixed. After vortexing the reagent sample mixture, the color produced was measured at 595 nm versus standards. The bovine serum albumin standards were prepared in the range of 0 to 800 µg $ml^{-1}$.

### 3.2.4 Assays of Antioxidative Enzymes

Approximately, 1 g of fresh leaf samples was homogenized using mortar and pestle, in 5 ml of ice-cold 50 mM phosphate extraction buffer (pH 7.6) containing 0.1 mM Na-EDTA. The homogenized samples were first centrifuged at 4600 *g* for 15 min, pellet discarded. Supernatant was centrifuged again at 15000 *g* for 15 min. Resultant supernatant was used for enzyme analysis. All operations until analysis were carried out at +4 °C. With the exception of SOD, all enzyme activities were measured in a final volume of 1 ml using various aliquots of the supernatants.

#### 3.2.4.1 Ascorbate Peroxidase Activity

Ascorbate peroxidase (AP) activity was determined as outlined by Cakmak (1994) by following decrease in absorbance of ascorbic acid at 290 nm (extinction coefficient 2.8 mM $cm^{-1}$) in a 1 ml of reaction mixture containing 50 mM Phosphate buffer (pH 7.6), 0.1 mM Na-EDTA, 12 mM $H_2O_2$, 0.25 mM ascorbic acid and the enzyme extract. Corrections were made for very low, non-enzymatic oxidation of ascorbic acid by $H_2O_2$.

### 3.2.4.2 Glutathione Reductase Activity

Activity of glutathione reductase (GR) was measured according to Cakmak and Marschner (1992) by monitoring the oxidation of NADPH at 340 nm (extinction coefficent 6.2 mM $cm^{-1}$). The reaction mixture (1 ml) contained 50 mM phosphate buffer (pH 7.6), 0.1 mM Na-EDTA, 0.5 mM oxidized glutathione (GSSG), 0.12 mM NADPH, and the enzyme extract. Corrections were made for the non-enzymatic oxidation of NADPH by recording the decrease at 340 nm without adding GSSG to the assay mixture.

### 3.2.4.3 Superoxide Dismutase Activity

Activity of superoxide dismutase (SOD) was assayed by a photochemical method described by Cakmak and Marschner (1992) and based on a SOD-inhibitable reduction of nitro blue tetrazolium chloride (NBT) by superoxide radicals. Assays were carried out under illumination in growth chamber. For the SOD assay, the reaction medium (5 ml) was consisted of 50 mM phosphate buffer (pH 7.6), 0.1 mM Na-EDTA, enzyme extracts (50-150 µl), 50 mM $Na_2CO_3$ (pH 10.2), 12mM L-methionine, 75 µM p-nitro blue tetrazolium chloride (NBT) and finally 2 µM riboflavin was added in glass vials and the reaction has been initiated by turning the lights on. The light intensity was about 700 µE $m^{-2}$ $s^{-1}$ and the assay lasted 15 min. The amount of enzyme extract that caused a 50 % decrease in the SOD-inhibitable NBT reduction was defined as 1 unit, at 560 nm.

### 3.2.4.4 Catalase Activity

Catalase (CAT) activity was determined according to Cakmak and Marschner (1992). The assay was based on the decrease in the absorbance of $H_2O_2$ recorded at 240 nm (extinction coefficient 39.4 mM $cm^{-1}$ ). The reaction medium (1 ml) contained 50 mM phosphate buffer (pH 7.6), 0.1 mM Na-EDTA, 100 mM $H_2O_2$ and the enzyme extract.

### 3.2.5 Measurement of NADPH-dependent $O_2^{\cdot-}$ Generation

Approximately, 2 g of fresh root sample was homogenized using mortar and pestle, in 5 ml of ice-cold 50 mM phosphate extraction buffer (pH 8.0) containing 0.1 mM Na-EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.3 % (w/v) polyvinylpyrrolidone. The homogenized samples were first centrifuged at 4600 *g* for 15 min, pellet discarded. Supernatant was centrifuged again at 15000 *g* for 15 min. Resultant supernatant was used for measurement of NADPH-dependent $O_2^{\cdot-}$ generation and NADPH oxidase activity. All operations were carried out at +4°C until analysis.

The assay of NADPH-dependent $O_2^{\cdot-}$ generation in the root extracts was carried out as described by Cakmak and Marschner (1988) with some modifications. In the present study we used NBT instead of cytochrome c, and we measured the rate of SOD-inhibitable NBT reduction in the presence of NADPH. Reaction mixtures in reference and sample cuvettes contained 50 mM phosphate buffer (pH 8.0) 0.1 mM EDTA, 1 µM KCN, 0.5 mM NBT, root extract and 50 µM NADPH in a total volume of 1 ml. Superoxide dismutase was added to the reference cuvette at a final concentration of 25 µg $ml^{-1}$ (100 U/100 µl). After a 1 min. preincubation the reaction was started by the addition of NADPH to both cuvettes, and the changes in absorbance was recorded at 550 nm for 5 min. Rates of $O_2^{\cdot-}$ generation was calculated using an extinction coefficient of 12.8 mM $cm^{-1}$ .

### 3.2.6 NADPH Oxidase Activity

NADPH oxidase activity was determined according to Cakmak and Marshner (1988). The assay was based on the oxidation of NADPH recorded at 340 nm (extinction coefficient 6.2 mM $cm^{-1}$), under same conditions with the NADPH-dependent $O_2^{\cdot -}$ generation, except that NBT and SOD was omitted from the reaction mixture.

# 4 RESULTS

## 4.1 Greenhouse Experiments

### 4.1.1 Shoot Dry Matter Production and Cadmium Tolerance Index

The first reaction of plants to Cd toxicity was the reduction of shoot elongation and leaf size. The development of brown patches on the basis of oldest leaves was also characteristic symptoms for Cd toxicity and occurred after the reduction of shoot elongation. Appearance time and severity of the symptoms differed among and within durum and bread wheat cultivars. When grown under Cd concentrations (20 mg $kg^{-1}$), durum wheat cultivars (Selcuklu, Balcalı-2000, Zenit, Meram, Yilmaz and Balcalı-85) showed earlier and more severe symptoms than the bread wheat cultivars (Aytin, Cetinel 2000, Yakar, Atay 85, AK702, Dagdas, Bolal, Bagci, ES 14, and 03KE12). Among the durum wheat cultivars, Balcalı-2000 and Yilmaz particularly affected from Cd toxicity, while Balcalı-85 and Meram were the least affected durum wheat cultivars. In the case of bread wheat cultivars, 03KE12 and Cetinel 2000 were the most tolerant and the most sensitive bread wheat cultivars.

Despite the large genotypic differences in development of Cd toxicity symptoms, the effect of Cd toxicity on shoot dry matter production among bread and durum wheat cultivars was not very distinct. The average decreases in shoot dry matter production due to Cd toxicity were 30 % in bread wheat and 31 % in durum wheat (Table 4.1).

Accordingly, average shoot tolerance index, expressed as the ratio of shoot dry weight at Cd supply to the shoot dry weight without Cd supply, were nearly same for bread and durum wheat genotypes. Nevertheless, the tolerance index showed an appreciable difference between the most and least affected cultivars after Cd application (Table 4.1). For example, Cd tolerance index of the most sensitive durum wheat cultivar Balcalı-2000 was 61 % while in the less affected cultivar Balcalı-85 this ratio was 85 %. A similar variation was also found between the bread wheat cultivars (Table 4.1).

**Table 4.1** Shoot dry matter production and Cd tolerance indices of 10 bread and 6 durum wheat genotypes grown for 34 days under greenhouse conditions with + Cd (20 mg Cd kg-1 soil) and without Cd application (-Cd). Cadmium tolerance index was calculated as the ratio of shoot (or root) dry weight at different Cd concentrations to that without Cd supply (control treatment). The data represent mean±SD of three independent replications.

| Genotypes | Leaf Symptoms[a] | Dry Matter Production (mg plant$^{-1}$) - Cd | Dry Matter Production (mg plant$^{-1}$) + Cd | Cd Tolerance Index (%) |
|---|---|---|---|---|
| *T.aestivum* | | | | |
| 03KE12 | 1 | 0.38 ± 0.23 | 0.31 ± 0.05 | 83 |
| Bagci | 1 | 0.37 ± 0.07 | 0.29 ± 0.09 | 79 |
| AK 702 | 2 | 0.39 ± 0.16 | 0.29 ± 0.16 | 74 |
| Dagdas | 2 | 0.41 ± 0.10 | 0.30 ± 0.24 | 74 |
| Aytin | 3 | 0.39 ± 0.13 | 0.28 ± 0.14 | 71 |
| Atay 85 | 1 | 0.36 ± 0.09 | 0.24 ± 0.10 | 67 |
| Bolal | 1 | 0.44 ± 0.33 | 0.29 ± 0.12 | 66 |
| Yakar | 1 | 0.36 ± 0.29 | 0.23 ± 0.19 | 64 |
| ES 14 | 1 | 0.32 ± 0.23 | 0.20 ± 0.15 | 63 |
| Cetinel 2000 | 1 | 0.38 ± 0.35 | 0.23 ± 0.01 | 61 |
| Average | | 0.38 | 0.27 | 70 |
| *T.durum* | | | | |
| Balcalı 85 | 1 | 0.34 ± 0.09 | 0.29 ± 0.09 | 85 |
| Meram | 1 | 0.36 ± 0.04 | 0.25 ± 0.18 | 70 |
| Selcuklu | 2 | 0.37 ± 0.21 | 0.25 ± 0.09 | 69 |
| Zenit | 1 | 0.35 ± 0.11 | 0.23 ± 0.05 | 67 |
| Yilmaz | 3 | 0.36 ± 0.09 | 0.24 ± 0.09 | 65 |
| Balcali 2000 | 4 | 0.29 ± 0.09 | 0.18 ± 0.12 | 61 |
| Average | | 0.34 | 0.24 | 69 |

[a] Severity of leaf symptoms of Cd toxicity developed as brown necrotic spots on the base of the oldest leaves (See Fig. 4.2): 1(slight) to 4 (severe)

Based on the results of the greenhouse screening experiment, the durum wheat cultivars Balcalı-2000 and Balcalı-85 were selected to use in the further experiments related to physiological effects of Cadmium toxicities in both cultivars.

## 4.2 Growth Chamber Experiments

### 4.2.1 Leaf Symptoms

As found in the greenhouse experiment in soil culture, also in the growth chamber experiment using nutrient solution culture, Balcalı-85 was much lesser affected from Cd toxicity then Balcalı-2000 as judged from the severity of leaf symptoms under Cd supply. The first visible reaction of both durum wheat cultivars to Cd application was the reduction of shoot elongation with increasing Cd supply (Fig 4.1). Associated with this observation development of necrotic brown patches on the older leaves was observed. There were no necrotic symptoms on younger leaves. The younger leaves displayed reduction in size due to Cd toxicity. Although both cultivars seemed to respond similarly to increasing Cd application, regarding the decrease in shoot length, they differed, however, greatly in development time and severity of necrotic patches on leaves. (Fig. 4.1 and 4.2) As indicated, Balcalı-2000 was more seriously affected by Cd toxicity than Balcalı-85. Necrotic patches developed very rapidly and thereafter older leaves totally collapsed with the duration of Cd application (Fig 4.2). In the case of Balcalı-85, necrotic patches on the older leaves were very slightly developed.


**Figure 4.1** Shoot growth of the durum wheat cultivars Balcalı-2000 (A) and Balcalı-85 (B) with increasing Cd application. After 13 days of growth without Cd application in nutrient solution, plants were treated for 5 days by increasing Cd concentrations (0.5-30 µM $CdSO_4$) in nutrient solution before harvest

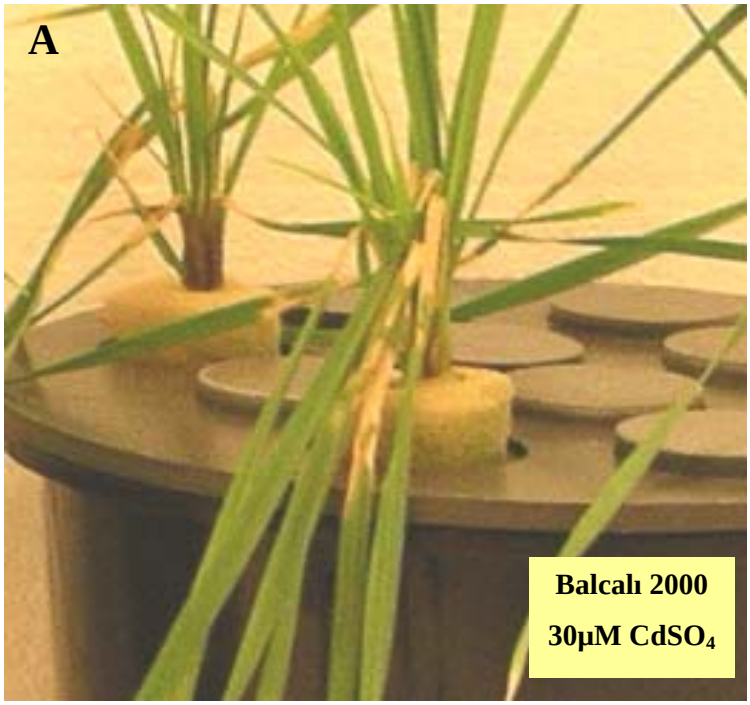



**Figure 4.2** Leaf symptoms of the durum wheat cultivars Balcalı-2000 (A) and Balcalı-85 (B) with increasing Cd application. After 13 days of growth without Cd application in nutrient solution, plants were treated for 5 days at 30 µM Cd concentration in nutrient solution before harvest

### 4.2.2 Dry Matter Production and Cadmium Tolerance Index

Both cultivars were severely affected in response to varying degree of Cd application. Continuous decrease was found in the dry weights of shoots and roots by increasing Cd application from 0 up to 30 μM (Table 4.2). However, theses decreases were more distinct in Balcalı-85 than in Balcalı-2000, especially in the case of shoot. With increasing Cd supply from 0 to 30 μM the shoot dry weight of Balcalı-2000 was decreased by 37 %, whereas the decrease in Balcalı-85 was 20 %, confirming the results obtained in the greenhouse experiment. Accordingly, the shoot tolerance index, expressed as the ratio of shoot dry weight at Cd supply to the shoot dry weight without Cd supply, was also greater in Balcalı-85, especially at the highest Cd application (Table 4.2).

In the case of root dry matter production, differences between two genotypes were very little (Table 4.2). Interestingly, at lower Cd treatments Balcalı-85 tended to be more sensitive to Cd toxicity regarding the root growth. However at the highest Cd supply Balcalı-85 appeared to be more tolerant. Overall, the differences in root dry matter production between genotypes are so small that no clear conclusions can be made. It seems likely that in both genotypes root growth was not differentially affected by Cd.

**Table 4.2** The effect of increasing Cd application on shoot and root dry weights and Cd tolerance index of 18 days-old two durum wheat cultivars, Balcalı-2000 and Balcalı-85. After growth for 8 days in nutrient solution without Cd application, plants were treated for 5 days by increasing Cd concentrations in nutrient solution before harvest. The data represent mean±SD of four independent replications.

| | Dry Matter Production | | Tolerance Index | |
|---|---|---|---|---|
| Cd supply | Balcali 2000 | Balcali 85 | Balcalı 2000 | Balcali 85 |
| (µM) | (g plant$^{-1}$) | | (%) | |
| | Shoot | | | |
| 0 | 0.37 ± 0.04 | 0.46 ± 0.02 | | |
| 0.5 | 0.35 ± 0.04 | 0.45 ± 0.04 | 93 | 99 |
| 2 | 0.33 ± 0.02 | 0.41 ± 0.11 | 87 | 89 |
| 10 | 0.30 ± 0.03 | 0.39 ± 0.05 | 79 | 84 |
| 30 | 0.24 ± 0.03 | 0.36 ± 0.05 | 63 | 80 |
| | Root | | | |
| 0 | 0.15 ± 0.00 | 0.21 ± 0.02 | | |
| 0.5 | 0.16 ± 0.02 | 0.20 ± 0.04 | 104 | 96 |
| 2 | 0.15 ± 0.02 | 0.19 ± 0.05 | 99 | 93 |
| 10 | 0.14 ± 0.01 | 0.19 ± 0.03 | 97 | 91 |
| 30 | 0.10 ± 0.01 | 0.15 ± 0.03 | 67 | 73 |

### 4.2.3 Cadmium Concentration and Content

Cadmium concentrations of both cultivars were significantly enhanced by increasing Cd application both in shoots and roots (Table 4.3). Cadmium was particularly accumulated in the roots of both cultivars. This accumulation was more pronounced in Balcalı-85. However, at each Cd supply, Balcalı 85 had much lower Cd concentration in shoot than Balcalı-2000. Generally, shoot Cd concentrations of Balcalı-2000 were nearly two-fold more than that of Balcalı-85. These results indicate that Balcalı-85 has better ability to keep Cd in roots and maintain lower concentrations of Cd in shoots. Similar to the results with Cd concentration, the total amount of Cd per plant (Cd content) was also enhanced by increasing levels of Cd application in both cultivars. At low concentrations of Cd (0.5 and 2 µM Cd) shoot content values of Balcalı-2000 were nearly twice as much as Balcalı-85 (Table 4.3). At the higher treatments of Cd the genotypic differences in shoot content of Cd became smaller (Table 4.3). In contrast to

shoot, the root contents of Cd were greater in Balcalı-85 compared to Balcalı-2000. These results clearly suggest that Balcalı-2000 translocated more Cd to shoots than Balcalı-85 did. To verify this suggestion Cd translocation index has been calculated by dividing total amount of Cdin shoots to the total amount of Cd in the plant (root+shoot). The results showed that in all Cd treatments the translocation index was almost two-fold higher in Balcalı-2000 than Balcalı-85.

In order to study the differences in the uptake and accumulation of Cd between both cultivars, which were treated with high levels of Cd (10 and 30 μM) for 3 days, short-term Cd accumulation was studied. The results are presented in Table 4.4. Correlated with previous results seen in Table 4.3, plant Cd concentrations (root+shoot) of Balcalı-85 were higher than Balcalı-2000, accumulating more Cd in the roots. However, Balcalı-2000 tended to accumulate more Cd in shoots (Table 4.4).

**Table 4.3** The effect of increasing Cd application on concentration and content (total amount) of Cd in shoots and roots of 18 days-old two durum wheat cultivars, Balcalı-2000 and Balcalı-85. After grown for 8 days in nutrient solution without Cd application, plants were treated for 5 days by increasing Cd concentrations in nutrient solution before harvest. The data represent mean±SD of four independent replications.

| | Cd Concentration | | Cd Content | | Translocation Index | |
|---|---|---|---|---|---|---|
| Cd supply | Balcali 2000 | Balcali 85 | Balcali 2000 | Balcali 85 | Balcali 2000 | Balcali 85 |
| (µM) | (mg kg-1 DW ) | | (µg plant-1) | | (%) | |
| | | | Shoot | | | |
| 0 | 0.1 ± 0.02 | 0.1 ± 0.04 | 0.1 ± 0.03 | 0.1 ± 0.07 | | |
| 0.5 | 10 ± 0.3 | 4 ± 0.5 | 13 ± 1.4 | 7 ± 0.7 | 33 | 14 |
| 2 | 20 ± 1.4 | 11 ± 1.2 | 26 ± 2.4 | 18 ± 6.3 | 22 | 12 |
| 10 | 23 ± 1.4 | 17 ± 1.9 | 29 ± 2.0 | 27 ± 4.8 | 12 | 7 |
| 30 | 33 ± 1.8 | 18 ± 6.2 | 30 ± 2.6 | 27 ± 10.2 | 9 | 5 |
| | | | Root | | | |
| 0 | 0.1 ± 0.03 | 0.1 ± 0.05 | 0.1 ± 0.02 | 0.1 ± 0.04 | | |
| 0.5 | 45 ± 8 | 58 ± 5 | 28 ± 2 | 46 ± 4 | | |
| 2 | 165 ± 12 | 173 ± 38 | 96 ± 7 | 130 ± 27 | | |
| 10 | 375 ± 40 | 449 ± 40 | 216 ± 24.1 | 340 ± 50 | | |
| 30 | 773 ± 46 | 917 ± 143 | 308 ± 41.3 | 546 ± 78 | | |

**Table 4.4** The effect of increasing Cd application on concentration and content (total amount) of Cd in shoots and roots of 16 days-old two durum wheat cultivars, Balcalı-2000 and Balcalı-85. After growth for 8 days in nutrient solution without Cd application, plants were treated for 3 days at 10 µM and 30 µM Cd concentrations in nutrient solution before harvest. The data represent mean±SD of four independent replications.

| | Cd Concentration | | Cd Content | | Translocation index | |
|---|---|---|---|---|---|---|
| | Cd 10 µM | Cd 30 µM | Cd 10 µM | Cd 30 µM | Cd 10 µM | Cd 30 µM |
| | (mg kg-1 DW ) | | (µg plant-1) | | (%) | |
| Cultivars | | | Shoot | | | |
| Balcali 2000 | 32 ± 0.9 | 41 ± 5.7 | 22 ± 1.7 | 28 ± 1.8 | 15 | 11 |
| Balcali 85 | 19 ± 1.5 | 24 ± 1.6 | 18 ± 3.6 | 20 ± 2.2 | 8 | 5 |
| | | | Root | | | |
| Balcali 2000 | 367 ± 48.9 | 770 ± 14 | 123 ± 14 | 262 ± 37 | | |
| Balcali 85 | 460 ± 155 | 1121 ± 151 | 212 ± 28 | 412 ± 8 | | |

### 4.2.4 CadmiumUptake

#### 4.2.4.1 Nutrient Solution

In order to determine the uptake rates of Cd in both cultivars, Cd concentration in nutrient solutions were measured during the course of 24 h at both 2µM and 10µM Cd treatments. Uptake solution has been sampled at different time intervals to calculate the amount of Cd depleted by the roots from the uptake solution. The results are shown in Fig 4.4 for the plants exposed to 2 µM Cd and Fig 4.5 for the 10 µM Cd. At 6 h after Cd exposure to 2µM Cd treatment Balcalı-2000 showed greater Cd uptake rate than Balcalı-85 (Fig. 4.3 A and B). Thereafter, no clear difference in Cd uptake rate was found between both cultivars (Fig 4.3 A). Similar results were also found for the cumulative uptake of Cd. As expected, the cumulative Cd uptake progressively increased with the duration of the Cd treatment (Fig 4.3 B).After 6 h exposure time, the genotypes remained more or less similar in their total uptake capacity for Cd (Fig. 4.3 B).

In the case of 10 µM Cd application, both cultivars were not different in their Cd uptake rate and cumulative Cd uptake (Fig 4.4 A and B). When compared to the Cd uptake results with 2 µM Cd treatment, both Cd uptake rate and cumulative Cd uptake were much greater with 10 µM. However, after 6 h of Cd treatment, the decrease in Cd uptake rate was much stronger at 10 µM Cd application. The marked increases in the cumulative Cd uptake during exposure to 2 µM Cd were not found at 10 µM Cd supply. These results indicate a saturation situation in Cd uptake and accumulation with 10 µM Cd application.

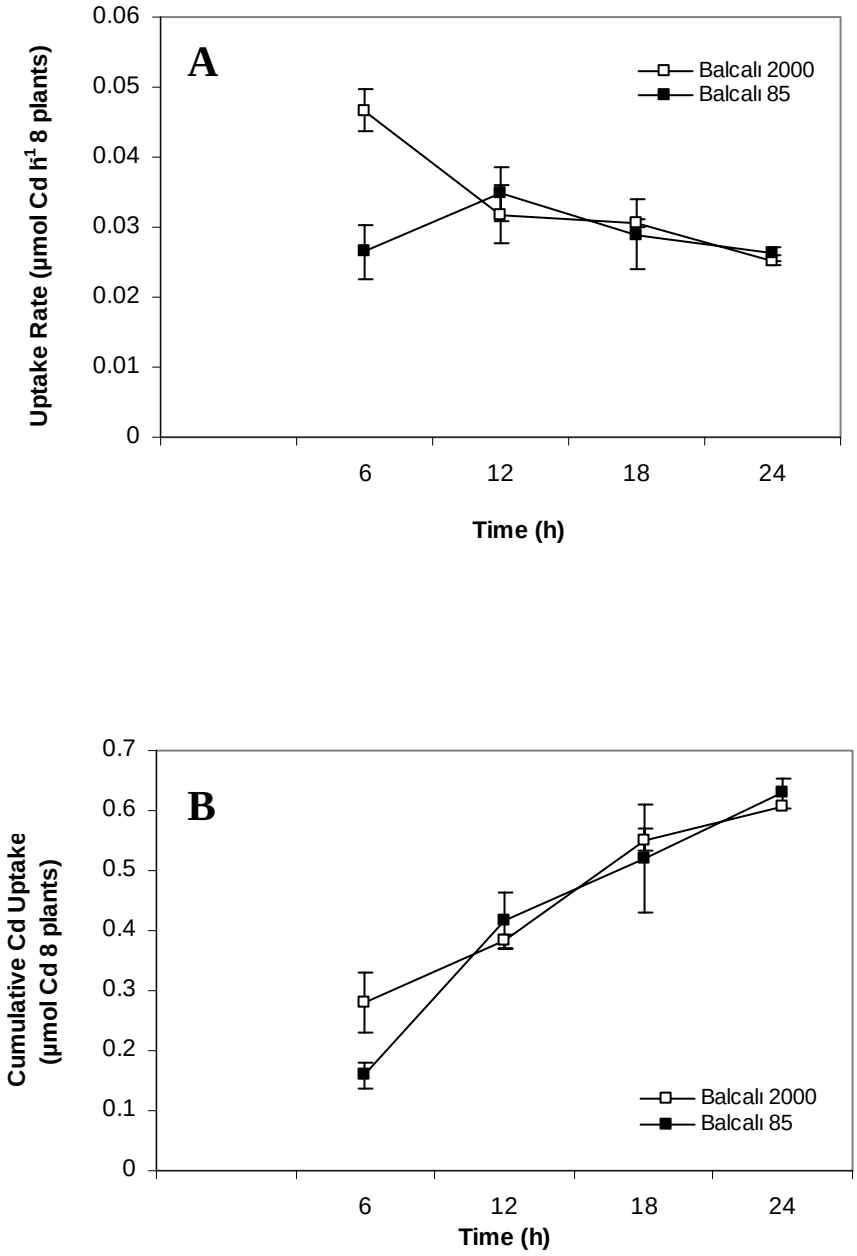


**Figure 4.3** Influence of exposure time on concentration of Cd in nutrient solutions of 8 days-old two durum wheat cultivars Balcalı-2000 and Balcalı-85. Plants were grown for seven days at in nutrient solution and treated with 2 µM Cd for 24 hours before harvest. Uptake rate represented as µmol Cd 8 plants per hour (A). Cumulative uptake rate represented as µmol Cd 8 plants (B). The data represent mean±SD of three independent replications.

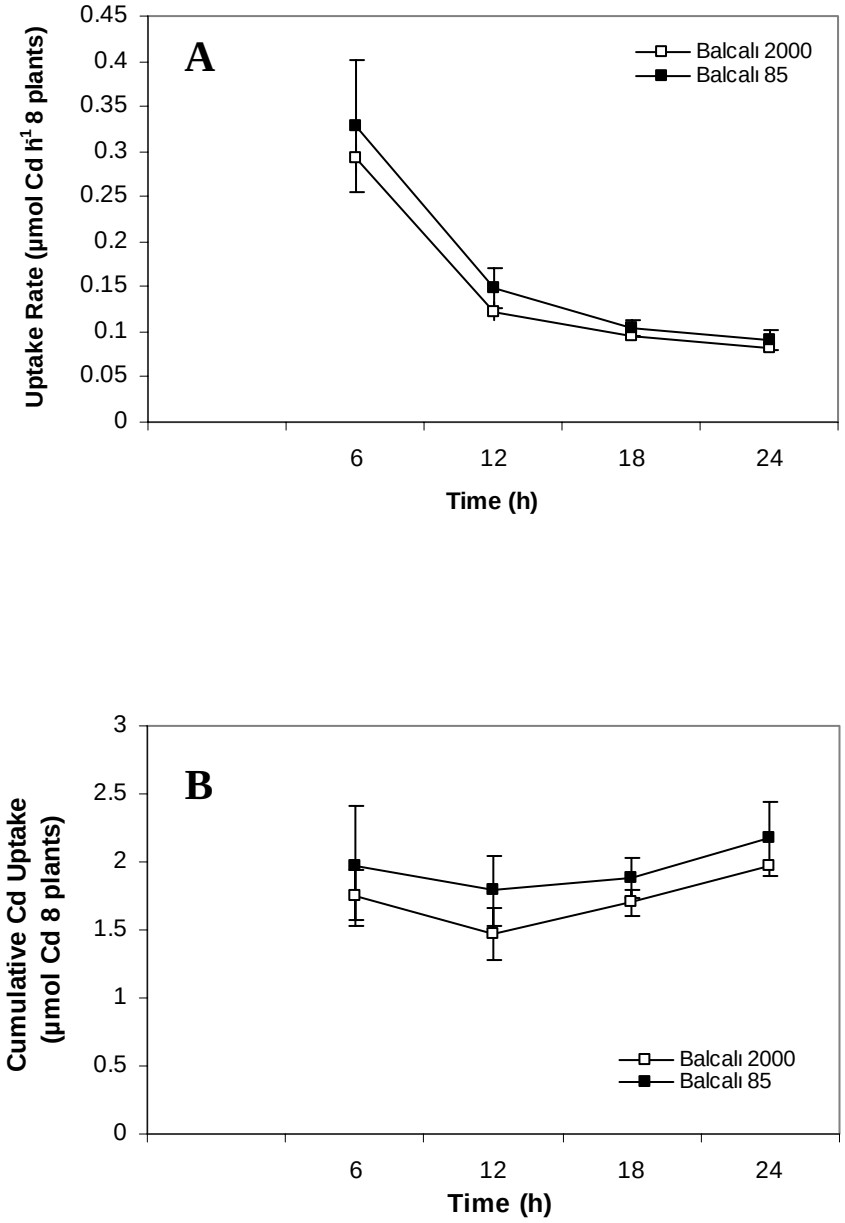


**Figure 4.4** Influence of exposure time on concentration of Cd in nutrient solutions of 8 days-old two durum wheat cultivars Balcalı-2000 and Balcalı-85. Plants were grown for seven days at in nutrient solution and treated with 10 µM Cd for 24 hours before harvest. Uptake rate represented as µmol Cd 8 plants per hour (A). Cumulative uptake rate represented as µmol Cd 8 plants (B). The data represent mean±SD of three independent replications.

### 4.2.5 Shoot Soluble Protein Concentration

At lower Cd treatments (0 to 2 µM), shoot soluble protein concentrations of both cultivars were relatively similar while at higher Cd treatments Balcalı-85 tended to show higher amount of soluble protein concentrations in shoot (Table 4.5). In general with increasing Cd supply there was an increasing and decreasing trend in shoot protein concentrations of Balcalı-85 and Balcalı-2000, respectively.

**Table 4.5** The levels of soluble protein in shoots of 18 days-old two durum wheat cultivars, Balcalı-2000 and Balcalı-85. Plants were treated for 5 days with increasing Cd concentrations in nutrient solution before harvest. The data represent mean±SD of four independent replications.

| | Genotypes | | | |
|---|---|---|---|---|
| Cd supply | Balcalı 2000 | *(%) | Balcalı 85 | *(%) |
| (µM) | (mg $g^{-1}$ TA) | | (mg $g^{-1}$ TA) | |
| 0 | 16.9 ± 1.1 | 100 | 17.7 ± 1.3 | 100 |
| 0.5 | 16.7 ± 0.3 | 99 | 18.9 ± 1.4 | 106 |
| 2 | 18.0 ± 1.3 | 107 | 21.3 ± 0.8 | 120 |
| 10 | 15.5 ± 1.2 | 92 | 19.9 ± 2.8 | 112 |
| 30 | 14.6 ± 2.0 | 87 | 19.1 ± 2.1 | 108 |

*% amount according to control treatment (0 µM $CdSO_4$)

### 4.2.6 Ascorbate Peroxidase

Ascorbate peroxidase (AP) activity of shoots expressed per mg protein, did not show a consistent change in Balcalı-2000 by increasing Cd treatments and remained more or less similar between the Cd treatments (Table 4.6). In the case of Balcalı-85 there was a clear decreasing trend in the AP activity with increase in Cd application. At lower Cd concentrations both cultivars were not clearly different, however at higher Cd treatments Balcalı-2000 had greater AP activity than Balcalı-85 (Table 4.6).

**Table 4.6** Changes in activity of ascorbate peroxidase in shoots of 13 days-old two durum wheat cultivars, Balcalı-2000 and Balcalı-85. Plants were grown for 5 days by increasing Cd concentrations in nutrient solution before harvest. The data represent mean±SD of four independent replications.

| Cd supply | Genotypes | | | |
|---|---|---|---|---|
| | Balcalı 2000 | | Balcalı 85 | |
| (µM) | AP (nmol $mg^{-1}$ Prt. $min^{-1}$) | (*%) | AP (nmol $mg^{-1}$ Prt. $min^{-1}$) | (*%) |
| 0 | 344 ± 31 | 100 | 371 ± 61 | 100 |
| 0.5 | 364 ± 19 | 106 | 312 ± 36 | 84 |
| 2 | 364 ± 25 | 106 | 299 ± 16 | 81 |
| 10 | 409 ± 63 | 119 | 289 ± 17 | 78 |
| 30 | 328 ± 34 | 95 | 218 ± 26 | 59 |

*% amount according to control treatment (0 µM $CdSO_4$)

### 4.2.7 Glutathione Reductase

Likewise AP activity, glutathione reductase (GR) activity of shoots exhibited an inconsistent trend in Balcalı-2000 by increasing Cd supply, while in the case of Balcalı-85 there was a decreasing trend in GR activity with increasing Cd application (Table 4.7). Under all treatments of Cd, the activity of GR was higher in Balcalı-2000 than Balcalı-85.

**Table 4.7** Changes in activity of glutathione reductase in shoots of 18 days-old two durum wheat cultivars, Balcalı-2000 and Balcalı-85. Plants were trated for 5 days by increasing Cd concentrations in nutrient solution before harvest. The data represent mean±SD of four independent replications.

| Cd supply | Genotypes | | | |
|---|---|---|---|---|
| | Balcalı 2000 | | Balcalı 85 | |
| (µM) | GR (nmol $mg^{-1}$ Prt. $min^{-1}$) | (*%) | GR (nmol $mg^{-1}$ Prt. $min^{-1}$) | (*%) |
| 0 | 78 ± 4 | 100 | 77 ± 4 | 100 |
| 0.5 | 82 ± 2 | 105 | 74 ± 5 | 96 |
| 2 | 68 ± 5 | 87 | 65 ± 5 | 84 |
| 10 | 80 ± 1 | 103 | 65 ± 4 | 84 |
| 30 | 61 ± 5 | 78 | 55 ± 4 | 71 |

*% amount according to control treatment (0 µM $CdSO_4$)

### 4.2.8 Superoxide Dismutase

Also in the case of superoxide dismutase (SOD), increasing Cd supply did not consistently affect the enzyme activity in Balcalı 2000, but the activity reduced very clearly in Balcalı-85. There was nearly 30 % decrease in SOD activity of Balcalı 85 when Cd was increased from 0 to 30 µM (Table 4.8).

**Table 4.8** Changes in activity of superoxide dismutase in shoots of 18 days-old two durum wheat cultivars, Balcalı-2000 and Balcalı-85. Plants were treated for 5 days by increasing Cd concentrations in nutrient solution before harvest. The data represent mean±SD of four independent replications.

| | Genotypes | | | |
|---|---|---|---|---|
| Cd supply | Balcalı 2000 | | Balcalı 85 | |
| (µM) | SOD (U mg protein$^{-1}$) | (*%) | SOD (U mg protein$^{-1}$) | (*%) |
| 0 | 6.6 ± 0.4 | 100 | 7.3 ± 0.9 | 100 |
| 0.5 | 6.2 ± 0.5 | 94 | 7.4 ± 0.8 | 101 |
| 2 | 5.7 ± 0.2 | 87 | 5.7 ± 0.4 | 77 |
| 10 | 5.9 ± 0.6 | 91 | 5.4 ± 0.6 | 74 |
| 30 | 7.3 ± 0.9 | 111 | 5.2 ± 0.3 | 71 |

*% amount according to control treatment (0 µM $CdSO_4$)

### 4.2.9 Catalase

Shoots of both cultivars responded with a noticeable decrease (% 35) in the activity of catalase (CAT) to increased Cd application (Table 4.9). In contrast to other antioxidative enzyme activities Balcalı-85 exhibited a higher catalase activity than that of Balcalı-2000 at all Cd treatments. When compared to the control treatment (no Cd application), the activity of CAT was significantly reduced in shoots of both cultivars upon exposure to 30 µM Cd. The activity of CAT decreased from 205 nmol mg$^{-1}$ prt. min$^{-1}$ to 133 nmol mg$^{-1}$ prt. min$^{-1}$ in Balcalı-2000 and from 218 nmol mg$^{-1}$ prt. min$^{-1}$ to 141 nmol mg$^{-1}$ prt. min$^{-1}$ in Balcalı-85 (Table 4.9).

**Table 4.9** Changes in activity of catalase in shoots of 18 days-old two durum wheat cultivars, Balcalı-2000 and Balcalı-85. Plants were treated for 5 days by increasing Cd concentrations in nutrient solution before harvest. The data represent mean±SD of four independent replications.

| | Genotypes | | | |
|---|---|---|---|---|
| Cd supply | Balcalı 2000 | | Balcalı 85 | |
| (µM) | CAT (nmol $mg^{-1}$ Prt. $min^{-1}$) | (*%) | CAT (nmol $mg^{-1}$ Prt. $min^{-1}$) | (*%) |
| 0 | 205 ± 6 | 100 | 218 ± 9 | 100 |
| 0.5 | 219 ± 17 | 107 | 224 ± 9 | 103 |
| 2 | 186 ± 12 | 91 | 194 ± 3 | 89 |
| 10 | 143 ± 8 | 70 | 191 ± 15 | 88 |
| 30 | 133 ± 19 | 65 | 141 ± 15 | 65 |

*% amount according to control treatment (0 µM $CdSO_4$)

### 4.2.10 Root Soluble Protein Concentration

Soluble protein concentrations in roots remained nearly constant at lower Cd applications (0.5 and 2 µM Cd) and than showed an increase at higher Cd treatments (10µM and 30µM Cd) (Table 4.10). In Balcalı-85 effect of increasing Cd treatment on root protein concentrations of Balcalı-85 was very variable, but resulted in an increase at all treatments (Table 4.10).

**Table 4.10** The levels of soluble protein in roots of 18 days-old two durum wheat cultivars, Balcalı-2000 and Balcalı-85. Plants were treated for 5 days by increasing Cd concentrations in nutrient solution before harvest. The data represent mean±SD of four independent replications.

| | Genotypes | | | |
|---|---|---|---|---|
| Cd supply | Balcalı 2000 | *(%) | Balcalı 85 | *(%) |
| (µM) | (mg $g^{-1}$ TA) | | (mg $g^{-1}$ TA) | |
| 0 | 0.94 ± 0.1 | 100 | 0.91 ± 0.0 | 100 |
| 0.5 | 0.95 ± 0.1 | 101 | 1.48 ± 0.0 | 163 |
| 2 | 1.00 ± 0.1 | 107 | 1.01 ± 0.1 | 111 |
| 10 | 1.59 ± 0.0 | 170 | 1.24 ± 0.1 | 137 |
| 30 | 1.27 ± 0.1 | 136 | 1.59 ± 0.0 | 175 |

*% amount according to control treatment (0 µM $CdSO_4$)

### 4.2.11 NADPH-dependent $O_2^{\cdot -}$ Generation

With exception of the highest Cd supply, increasing Cd treatments enhanced NADPH dependent $O_2^{\cdot -}$ generation in roots of Balcalı-2000. A similar trend was also found in roots of Balcalı 85. At the highest Cd supply the rate of $O_2^{\cdot -}$ generation in both cultivars was not affected when compared to the rate found at control treatment (Table 4.11).

In Balcalı-2000, NADPH oxidase activity was gradually suppressed by increasing Cd concentrations, while in Balcalı-85, there was first an inconsistent change until 10 µM Cd supply, and then at 30 µM Cd a sudden depression was noted (Table 4.11). At 30 µM Cd treatment, both cultivars displayed a considerable decrease in the activity of NADPH oxidase, which was about 73 % in Balcalı-2000 and 80 % in Balcalı-85 (Table 4.11).

**Table 4.11** Levels of NADPH-dependent $O_2^{\cdot-}$ generation and changes in the activity NADPH oxidase in roots of 18 days-old two durum wheat cultivars, Balcalı 2000 and Balcalı 85. Plants were treated for 5 days by increasing Cd concentrations in nutrient solution before harvest. The data represent mean±SD of four independent replications.

| | Genotypes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Cd supply | Balcalı 2000 | | Balcalı 85 | | Balcalı 2000 | | Balcalı 85 | |
| (µM) | O2.- production rate (nmol mg-1 Prt. min-1) | (*%) | O2.- production rate (nmol mg-1 Prt. min-1) | (*%) | NADPH oxidase (nmol mg-1 Prt. min-1) | (*%) | NADPH oxidase (nmol mg-1 Prt. min-1) | (*%) |
| 0 | 5.4 ± 0.5 | 100 | 3.1 ± 0.5 | 100 | 23.3 ± 2.5 | 100 | 40.7 ± 8.2 | 100 |
| 0.5 | 6.2 ± 0.8 | 116 | 2.4 ± 0.6 | 78 | 24.1 ± 1.3 | 103 | 33.6 ± 0.4 | 82 |
| 2 | 6.2 ± 1.4 | 116 | 4.5 ± 0.9 | 146 | 11.1 ± 1.0 | 48 | 45.9 ± 2.3 | 113 |
| 10 | 6.9 ± 1.1 | 128 | 4.6 ± 0.4 | 149 | 6.7 ± 0.9 | 29 | 53.4 ± 7.0 | 131 |
| 30 | 5.4 ± 0.3 | 100 | 3.2 ± 0.2 | 105 | 6.3 ± 0.3 | 27 | 8.2 ± 1.5 | 20 |

*% amount according to control treatment (0 µM CdSO4)

# 5 DISCUSSION

## 5.1 Leaf Symptoms and Growth

Reduction of the shoot length and the decrease in biomass of plants are the first indications of Cd toxicity. These indications could be observed in few days after Cd application. Rapid development of chlorosis, occurrence of brown patches, especially at the base of the older leaves, and browning of the roots were the visual syptoms in the Cd-treated plants. Similar observations were also made by Cosio et. al., (2005). In their study, increasing Cd concentrations (from 5 to 200 μM Cd) caused chlorosis, followed by expansion of necrosis and leaf rolling in willows. Additionally, in *Elodea canadensis,* a thinner stem, less expanded leaves with partial bleaching of green tissues and 40 % internode shortening were observed in response varying degrees of Cd treatments when compared to the shoot of control plants (Vecchia et al., 2005). In the present study, we made similar observations in two durum wheat genotypes under Cd toxicity. Among the genotypes, Balcalı-2000 was more sensitive to Cd toxicity, and the toxicity symptoms developed much earlier in Balcali-2000 than Balcalı-85. The development of necrotic patches on the basis of older leaves was one of the early responses of durum wheats to Cd toxicity (Fig 4.1 and 4.2). Although there was a great difference in development time and severity of Cd toxicity symptoms between two genotypes, there was, however, a smaller difference in Cd-dependent decreases in dry matter production (Table 4.2). Based on these results described above, Balcalı-85 was ranked as a Cd-tolerant genotype while Balcali-2000 was considered as a sensitive genotype.

Leaf chlorosis and necrosis under Cd stress could be a result of inhibited chlorophyll synthesis and/or chlorophyll degradation. Cadmium causes reduction in formation of 5-aminolaevulinic acid and protochlorophyll reductase which are critical parts in biosynthesis of chlorophyll (Stobart et. al., 1985). Moreover, lipid peroxidation associated with degradation of chlorophyll is another consequence of Cd toxicity (Foyer et. al., 1997). Cadmium toxicity causes the gradual reduction in the concentrations of photosynthetic pigments *chl* a, *chl* b and carotenoids, and consequently restricts activity of photosynthesis (Rai et. al., 2005). In *Phyllanthus amarus* plants Cd toxicity resulted in a 12-fold decrease in photosynthesis activity, and this was discussed as the reason for the reduced biomass production in Cd-treated plants (Leon et al., 2002).Also, Rai et. al., (2005) reported almost 80 % decrease in plant fresh and dry weights in response to Cd toxicity. In the present study, the decreases in the shoot dry weights with increasing Cd application were more obvious in Balcalı-2000 than that of Balcalı-85. In contrast to shoot growth, the root growth of Balcali-85 was affected by Cd toxicity slightly higher than in Balcali-2000 (Table 4.2). The reason for higher susceptibility of roots in Balcali-85 compared to shoot growth could be related to greater accumulation of Cd in roots, especially under low Cd treatments (Table 4.2; and see related discussion below). Also Cd affects ultracellular structure of meristematic cells, changing the ribosomal RNA precursor biosynthesis which possibly contributes to reduction in root growth (Marcano et. al., 2002). Roots, the first site contacting with Cd, have greater capacity to accumulate Cd than shoots. Therefore, a greater sensitivity of roots to Cd toxicity than shoots is generally observed (Grant et. al. 1998). Similarly, we found that when compared to shoots, the total amount of Cd accumulated in the roots was approximately 50-fold more in Balcalı-85 and 23-fold more in Balcalı-2000 at the highest Cd application (Table 4.3 and 4.4).

## 5.2 Cadmium Uptake and Translocation

Although Cd is not required as an essential nutrient in higher plants, the bioaccumulation index of Cd may exceed other elements (Kabata-Pendias and Pendias, 1992). Therefore, tolerance to Cd in higher plants can be related to i) the distribution of

Cd ions within the plant, ii) the transport rate of the metal from roots to shoots, and iii) formation of Cd and Cd-binding peptide complexes in roots and shoots (Hart et. al., 1998; Arao et. al., 2003; Stolt et. al., 2003).

In the present study, there was no significant difference in total amount of Cd in between the two cultivars (Balcalı-2000 and Balcalı-85). However, the difference in root Cd concentrations was more apparent between the two cultivars. This result indicates that exclusion of Cd during root uptake cannot be an explanation for the observed genotypic variation. In many plant species including wheat, maize and barley, Cd uptake at the root surface has been characterized (Grant et. al., 1998; Hart et. al., 1998). As demonstrated by Hart et. al., (1998), the difference in accumulation of Cd in grains of durum and bread wheats, was not due to the differential Cd influx rates in roots. Greger and Löfstedt, (2004) demonstrated that the net uptake of Cd in spring bread wheat was lower than the winter bread and durum wheats. In the present study, one of the most striking differences between Balcalı-2000 and Balcalı-85 was the shoot Cd concentration. Under all Cd applications, shoot accumulation of Cd in Balcalı-2000 was almost 2-fold more than that of Balcalı-85 (Table 4.3 and 4.4). As suggested by Stolt et. al., (2003), differential xylem loading or transport in durum wheat might be the main reasons of the genotypic variation found in our study. The Cd-sensitive cultivar Balcalı-2000, appears to translocate more Cd to shoots than the Cd-tolerant cultivar, Balcalı-85. The difference in the root-to-shoot translocation between two genotypes was also found under very low, environmentally relevant concentrations (e.g., 0.5 and 2 μM; Table 4.3).

At the end of the 3$^{rd}$ day of exposure to Cd, there was still a large difference in translocation rate between two cultivars which points out ability of Balcalı-85 to retain Cd at the root level. Parallel to our results, in pea, the amount of Cd taken up by roots and transferred to the shoots was relatively low in genotypes which were less sensitive to Cd (Metwally et. al., 2005). In a previous study conducted by Greger and Löfstedt, (2004), there was a significant correlation between Cd translocation index and grain Cd level. Hence, they suggested that translocation of Cd from root to shoot, and shoot Cd concentration may be an important factor for the accumulation of Cd in grains. Based on these results it can be suggested that Balcali-2000 eventually contains more Cd in

grain than Balcali-85. This is an important speculation and needs to be studied in future studies.

Our results demonstrate that Cd retention in roots is an important mechanism in Cd tolerance. The ability of genotypes to retain Cd in roots can contribute to reduced accumulation of Cd in grain which is of great importance for human health. As indicated above, Balcali-2000 seems to be a genotype having higher capacity to accumulate Cd in grain. Accordingly, the variation in Cd translocation to the seeds in different soybean genotypes has been proposed as a major factor involved in determining resistance to toxicity (Arao et. al., 2003).

Reduction of Cd transport from roots into shoot should be influenced by sequestration of the metal in the roots. This process can be regulated mostly by two ways: formation of phytochelatin-Cd complexes (Grill et. al., 1985) and compartmentalization of Cd in the vacuole (Rauser; 1995). It has been suggested that vacuolar compartmentalization of Cd may be a more effective mechanism in the inhibition of long distance transport throughout the plant, rather than Cd binding to phytochelatins in xylem translocation to shoots (Hart et. al., 1998). Based on these findings it can be suggested that the formation of PC-Cd complexes in roots and their compartmentalization in vacuoles might be a plausible explanation for the reduced transport of Cd from roots into shoot in Balcali-85.

## 5.3 Antioxidative Defense Enzymes

Under metal stress conditions, changes in antioxidative enzyme activities play an important role in metal tolerance. Among the antioxidative enzymes, activities of ascorbate peroxidase (AP), glutathione reductase (GR) and superoxide dismutase (SOD) decreased in Balcalı-85 whereas in Balcalı-2000 there was an inconsistent trend upon Cd supply (Table 4.6, 4.7, and 4.8). However, at the highest amount of Cd exposure, the activities of the antioxidative enzymes were clearly depressed in both genotypes, possibly due to inhibited protein synthesis or inactivation of enzymes by binding of Cd

to SH-groups of the enzymes (Ouzounidou et. al., 1997). For example, following Cd treatment SOD activity was shown to be reduced or remained at the control level in both roots and leaves in two wheat cultivars. In both wheat cultivars, AP activity was always higher in roots than leaves. In the case of leaves, AP activity was induced in the tolerant genotype and did not change in the sensitive cultivar (Milone et.al., 2003). Variation in response to Cd toxicity was also observed in *Arabidopsis thaliana*. Cadmium-resistant type, which was developed from a Cd-sensitive wild-type had higher activities of SOD, AP and GR (Cho and Seo, 2005). An increase in activity of AP and GR was observed in the leaves of *Phaseolus vulgaris* within 24 hours of exposure (Smeets et. al., 2005). Schützendübel et. al., (2002) demonstrated that in roots of *Populus canescens*, activity of antioxidative enzymes increased following 12 h of exposure, and thereafter resulted in a rapid decrease. Based on all these results from literature it seems that the results on Cd-dependent changes in antioxidative enzymes are very controversial which could be related to the rate and duration of Cd applied, plant genotype and age of plant tissue examined etc. In addition, it is important to focus on the isoforms of these enzymes and their localisation at cellular level (e.g., measurement of mitochondrial or chloroplastic forms of the enzymes) in future studies.

Among the enzymes studied, catalase (CAT) is localized in peroxisomes and responsible for $H_2O_2$ scavenging. In contrast to the other antioxidative enzyme activities, CAT activity was particularly decreased in both Balcalı-2000 and Balcalı-85. Similarly, a reduced CAT activity was found in leaves of *Arabidopsis thaliana* (Cho and Seo, 2005) after Cd exposure. In both roots and shoots of *Bacopa monnieri,* a significant decline in CAT activity was reported after 50 μM Cd applications for 48 hours (Singh et. al., 2006). A decline in the CAT activity was also shown in pepper plants (León et. al., 2002). These results indicate that CAT is highly sensitive enzyme to Cd treatments. The reason for such high sensitivity to Cd could not be understood and may be related to inhibition of protein synthesis or binding of Cd to enzyme complex.

## 5.4 NADPH-Dependent $O_2^{\cdot -}$ Generation

Since roots are the first parts of plants that are exposed to excess amounts of Cd, therefore accumulated majority of the metal supplied, and thus interactions of Cd with membrane lipids and proteins mainly occurs at the root level. The membrane-bound NADPH oxidases are activated by different biotic and abiotic stresses. Cadmium toxicity can also result in structural impairments in cell membranes with a concominant activation of membrane-bound NADPH oxidase. Involvement of an NADPH oxidase-like enzyme has been suggested in the case of oxidative burst in cultured tobacco cells (Olmos et. al., 2003). It has been demonstrated that impairments in integrity of root cell membranes under Zn deficiency, Cu deficiency and toxicity were related to enhanced activity of $O_2^{\cdot -}$ generating NADPH oxidase (Cakmak and Marschner, 1988; Quartacci et. al., 2001). In the present study, enhanced formation of superoxide radical ($O_2^{\cdot -}$) by increasing Cd application was demonstrated in both cultivars (Table 4.11). In the roots of Balcalı-85 a comparable pattern in NADPH oxidase activity was displayed, indicating that enhanced activity of NADPH oxidase by Cd exposure may be a possible reason for Cd-induced $O_2^{\cdot -}$ formation. At 30 μM Cd supply, despite the induced $O_2^{\cdot -}$ production, the reduction in the enzyme activities of Balcalı-2000 and Balcalı-85 may be due to structural modification in membranes caused by Cd exposure. Another possible mechanism may be an inhibitory effect of Cd on NADPH oxidase. Competitive transport interaction of $Zn^{+2}$ and $Cd^{+2}$ was demonstrated at the root plasma membrane in durum wheat (Hart et. al., 2002). Since chemical properties of both ions are similar, inhibitory effect of Zn on the activity of NADPH oxidase (Cakmak, 2000), can be suggested as a mechanism for Cd-induced inactivation of NADPH oxidase in the present study.

# 6 CONCLUSIONS

The present work demonstrated existence of a large variation in tolerance to Cd toxicity between durum wheat cultivars. Balcalı-2000 is particularly affected by increasing Cd treatments, even at low levels of Cd treatments. Based on the leaf symptoms together with the results of tolerance index, Balcalı-2000 has been classified as Cd-sensitive cultivar, whereas Balcalı-85 ranked as Cd-tolerant cultivar.

Alterations in the antioxidant enzyme activities due to Cd toxicity were thought as a possible explanation for the observed genotypic differences in Cd tolerance between both cultivars. But, this was not the case in this study; the antioxidative defense systems remained ineffective in explanation the differential Cd tolerance found. However, as Balcalı-2000 accumulated more Cd in shoots and the enzyme activities appeared to be slightly increased, it can be suggested that Balcali-2000 possibly stimulates production of ROS. The reductions in the activities of antioxidative enzymes in Balcalı-85 indicate that tolerance characteristic of this cultivar can not be attributed to antioxidative defense mechanisms. Analysis of other parameters including, antioxidant levels, $H_2O_2$ accumulation, lipid peroxidation as well as identification of enzyme isoforms is needed for better understanding of the role of antioxidants in Cd tolerance described in the present work.

Variation in Cd concentration and content was the most distinct difference involved in differential expression of Cd tolerance between 2 durum wheats. In the shoots of Balcalı-2000, the amount of Cd was appeared to be 2 fold more than that of Balcalı-85, indicating a potential mechanism for retention of Cd in the root of Balcalı-85. In selecting genotypes with low levels of Cd in shoot (and also in grain), a special

attention should be paid to the capacity of the genotypes in retaining Cd in the roots like in Balcali-85. Such genetic ability of genotypes can also improve ability of genotypes to tolerate toxic effects of Cd in plants in shoots.

In cereal crops, heavy metal retention in roots and shoot is desirable because these parts are not consumed as food. It has been demonstrated that restriction of Cd transport from roots to shoots reduces grain Cd concentration much more than in leaves (Grant et. al., 1998). Therefore, the genotypic variation in internal Cd distribution between shoots and roots is especially crucial. Detoxification of Cd by complexion with peptides such as, phytochelatins or metallothioneins at the root level, or compartmentalization to the vacuole may be the reasons for Cd tolerance in Balcalı-85, and need to be elucidated in further studies. Moreover, Cd-responsive genes expressed in roots of Balcali-85 can be identified and used as markers in breeding and genetics programs, therefore should be studied in future.

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# APPENDIX

## Chemicals

All chemicals and standart solutions were supplied by Merck (Germany), SIGMA (USA), Fluka (Switzerland), Applichem (Germany) and Riedel de Häen (Germany).

## Equipment

| | |
|---|---|
| Autosampler for ICP: | Varian, SPS 3, AUSTRALIA |
| Balance: | Sartorius, CP 224 S, GERMANY<br>Sartorius, CP 3202 S, GERMANY |
| Centrifuge: | Kendro Lab. Prod., Heraeus Multifuge 3 S-R, GERMANY |
| Dispenser: | Brand, Seripetter, GERMANY<br>Brand, Dispensette, GERMANY |
| Distilled water: | Millipore, Elix-S, FRANCE<br>Millipore, MilliQ, Academic, FRANCE |
| Mill: | Fritsch, Vibrating Cup Mill, pulverisette 9, GERMANY |

| | |
|---|---|
| Muffle Furnace: | Nabertherm, Controller B 170, GERMANY |
| Ice machine: | Scotsman Inc., AF20, USA |
| Incubator: | Memmert, ULE 700, GERMANY<br>Memmert, ULE 600, GERMANY |
| Inductively coupled plasma-optical emission spectroscopy (ICP-OES): | Varian, Vista-Pro ccd, AUSTRALIA |
| Magnetic stirrer: | IKA®-WERKE, GERMANY<br>VELP Scientifica, Microstirrer, ITALY |
| Microliter Pipette: | Gilson, Pipetman, FRANCE<br>Eppendorf, GERMANY |
| pH meter: | Hanna Instrument, p213, Microprocessier pH meter, ROMANIA |
| Spectrophotometer: | Varian, Cary 300 BIO, UV-Visible, AUSTRALIA |
| Ultrafreezer: | - 80 °C Thermo, GERMANY |