EFFECT OF CADMIUM ON GROWTH AND ANTIOXIDANT ENZYMES IN TWO BARLEY CULTIVARS

by

MELİS TİRYAKİOĞLU

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EFFECT OF CADMIUM ON GROWTH AND ANTIOXIDANT ENZYMES IN TWO BARLEY CULTIVARS

APPROVED BY:

Prof. Dr. İsmail Çakmak ………………………….

(Dissertation Supervisor)

Prof. Dr. Rıfat Derici ………………………….

Assoc. Prof. Zehra Sayers ………………………….

DATE OF APPROVAL: ………………………….

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ABSTRACT

Cadmium (Cd) pollution is a growing environmental problem affecting human health and crop production. One strategy to minimize adverse affects of Cd toxicity on crop production is to develop plant genotypes having higher genetical ability to tolerate Cd toxicity. In the present MSc study, using two barley (*Hordeum vulgare*) cultivars (Tokak and Hamidiye) nutrient solution experiments were conducted to study genotypic variation in tolerance to Cd toxicity based on i) development of leaf symptoms and lipid peroxidation, ii) decreases in dry matter production, iii) Cd uptake and accumulation and iv) changes in antioxidative defence system in leaves (i.e., superoxide dismutase, ascorbate peroxidase, glutathione reductase, catalase, ascorbic acid and non-protein SH-compounds). Plants were grown in nutrient solution under controlled environmental conditions, and subjected to increasing concentration of Cd (e.g., 0, 15, 30, 60 and 120 μM Cd) for different periods.

The results obtained showed that among the barley cultivars Hamidiye is particularly sensitive to Cd as judged from the severity and development time of Cd toxicity symptoms on leaves. Within 48 h Hamidiye developed very rapidly and severely leaf symptoms of Cd toxicity while in Tokak the leaf symptoms of Cd toxicity appeared only slightly. Hamidiye also tended to show stronger decreases in growth caused by Cd supply for 48 h. Cadmium supply enhanced the level of lipid peroxidation in Hamidiye, but remained without effect in Tokak. The differences in sensitivity to Cd between Tokak and Hamidiye were not related to Cd concentrations in roots or shoots. Both barley cultivars were more or less similar in both concentration and accumulation (amount of Cd per plant) of Cd. The activities of enzymes involved in detoxification of hydrogen peroxide in chloroplasts and the activity of

superoxide dismutase, scavenger of superoxide radical, were markedly enhanced in Hamidiye by increasing Cd supply. However, in the case of Tokak there was either only a slight increase or no change in the levels of the enzymes. Interestingly, in both barley cultivars the activity of catalase was not influenced by Cd supply, indicating a particular affect of Cd on defence enzymes located in chloroplasts.

The results indicate existence of a large genotypic variation between barley cultivars for Cd tolerance. The differential Cd tolerance found in the barley cultivars was not related to uptake or accumulation of Cd in plants, indicating importance of internal mechanisms in expression of differential Cd tolerance in barley. Particular increases in antioxidative mechanisms in Cd-sensitive barley cultivar Hamidiye as a response to increasing Cd supply suggest that high Cd sensitivity of Hamidiye is related to enhanced production and oxidative attack of reactive oxygen species (ROS).

Keywords: Cadmium, barley cultivars, reactive oxygen species, oxidative stress and antioxidative enzymes.

ÖZET

Kadmiyum (Cd) kirlenmesi, giderek yaygınlık gösteren ve insan sağlığını ve bitkisel üretimi etkileyen bir çevresel problemdir. Kadmiyum toksitesine karşı yüksek düzeyde bir genetiksel dayanıklılık gösteren genotiplerin geliştirilmesi, bitkisel üretim üzerinde Cd toksitesinin olumsuz etkilerini azaltmak için dikkate alınabilecek bir strateji olarak görülebilir. Bu yüksek lisans çalışmasında iki arpa çeşidi (Tokak and Hamidiye) kullanılarak arpada Cd toleransının düzeyi çalışıldı. Denemeler besin çözeltisi ortamında yürütüldü ve çeşitlerin Cd toleransı i) yaprak belirtilerinin gelişimi ve lipid peroksidasyonu, ii) kuru maddede azalmalar, iii) Cd alımı ve bitkide akümülasyonu ve iv) yapraklarda antioksidatif savunma sistemlerindeki değişiklikler (superoksit dismutaz, askorbat peroksidaz, glutation redüktaz, katalaz, askorbik asid ve SH-grupları) düzeyinde belirlendi.

Kadmiyum toksisitesi semptomlarının yapraklardaki şiddeti ve gelişme hızı dikkate alındığında, arpa çeşitlerinden Hamidiye'nin Cd toksitesine çok duyarlı olduğu saptanmıştır. Kadmiyum uygulanmasıyla, ilk 48 saat içerisinde Hamidiye'nin yapraklarında çok hızlı ve şiddetli Cd toksisitesi semptomları görülürken, Tokak'ta bu semptomlar cok daha hafif görülmüştür. Kadmiyum Hamidiye'de lipid peroksidasyonun seviyesini arttırken Tokak'ta bir değişime yol açmamıştır. Tokak ve Hamidiye arasındaki kadmiyum toksitesine karşı farklı duyarlılıkta, kök ve yapraklardaki Cd konsantrasyonun rolü olmadığı saptanmıştır. Her iki arpa çeşidinde de Cd konsantrasyon ve birikiminin (bitki başına toplam Cd miktarı) benzer olduğu bulunmuştur. Kloroplasttaki hidrojen peroksit detoksifikasyonunda görev alan enzimlerin aktiviteleri ve superoksit radikalinin detoksifikasyonunu sağlayan superoksit dismutaz aktivitesi Cd uygulamalarıyla Hamidiye'de belirgin artış göstermiştir. Buna karşın Tokak'ta, enzim seviyelerinde ya hiç

artış gözlenmemiş ya da çok az bir artış görülmüştür. Her iki arpa çeşidinde de, katalaz aktivitesi Cd uygulamasından etkilenmemiştir. Bu sonuçlar, kadmiyumun özellikle kloroplastaki savunma enzimlerinin aktivitesini etkilediğini göstermektedir.

Sonuçlar, iki çeşidin kadmiyum toksitesini tolere edebilme özelliği açısından aralarında büyük bir genetiksel farklılığın olduğunu ortaya koymuştur. Kadmiyum toleransı açısından iki çeşit arasındaki farklılığın Cd alınımı ve birikimi ile ilgili olmadığı saptanmıştır. Bu da içsel mekanizmaların arpada Cd toleransında önemli olduğunu işaret etmektedir. Kadmiyum toksitesine çok duyarlı olan Hamidiye'de antioksidatif mekanizmasının Cd uygulamalarıyla artışa uğraması, Hamidiye'de reaktif oksijen türevlerinin üretildiğini ve bir oksidatif tahribatın ortaya çıktığını göstermektedir.

Anahtar Kelimeler: Kadmiyum, arpa çeşitleri, reaktif oksijen türevleri, oksidatif stres ve antioksidatif enzimler.

To my family

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ABBREVIATIONS

AP: Ascorbate peroxidase

AA: Ascorbic acid

Cd: Cadmium

DHA: Dehydroascorbate

DHAR*az*: Dehydroascorbate reductase

DW: Dry weight

FW: Fresh weight

GR: Glutathione reductase

GSH: Reduced glutathione

GSSG: Oxidised glutathione

H_2O_2: Hydrogen peroxide

$OH^{\cdot}$: Hydroxyl radical

MDHAR*az*: Monodehydroascorbate reductase

MDHA: Monodehydroascorbate

μg: microgram

mg: milligram

$NAD(P)^+$: Nicotinamide adenine dinucleotide

ROS: Reactive oxygen species

SOD: Superoxide dismutase

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

$^1O_2$: Singlet oxygen

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1. INTRODUCTION

Pollution of the environment with heavy metals is a growing concern, and generally occurs in well-developed countries. Environmental pollution with Cd is mainly caused by mining and smelting, dispersal of sewage sludge and the use of phosphate fertilisers (Chaney et al., 1998; Mejare and Bülow, 2001). At very high concentrations, Cd adversely affects plant growth and human health (Prasad, 1995; Grant et al., 1998). Entrance of Cd to human body occurs predominantly by food chain. According to FAO/WHO, maximum tolerable Cd intake is between 400 to 500 μg Cd per week. Cadmium exerts several toxic effects in lungs, livers and especially in kidneys. In Japan, there was high environmental exposure to Cd (Cupit et al., 2002; Vahter et al., 2002). Increases in Cd related diseases in 1960's in Japan awakened world awareness to Cd and its toxic effects (Jackson and Alloway, 1995). Heavy metal contamination of agricultural soils has also been an important problem in China (Siamwalla, 1996; Li et al., 1997; Chen et al., 1999).

Although Cd is not an essential mineral nutrient for plants, the metal can be taken up at very high amounts by crop plants including cereals, potatoes, vegetables and fruits (Grant et al., 1998) and in most cases causes inhibition of plant growth (Prasad, 1995; Sanita di Toppi et al., 1999). Investigations have been carried out to define the factors that affect plant availability of soil Cd, and health risk of food Cd (Chaney et al., 1998). In cultivated areas, the limit of total Cd concentration has been reported to be 3 mg kg^{-1} per kg soil whereas in the uncultivated areas it's around 0.1 mg kg^{-1} (Alloway, 1995). However, in areas subjected to mining, the concentration can be higher varying from 100 to 600 mg kg^{-1} dry weight (Ernst and Neilssen, 2000; Lombi et al., 2000). Cadmium accumulation in soil

by application of sewage sludge occurs mostly in the topsoil (Lombi et al., 2000) and the Cd existing in the topsoil can remain for 1000 years. The amount of Cd found in cultivated areas all over the world was 1100 tons in 1960, and it increased to 20200 tons in 1990 (Nriagu, 1998).

Cadmium, when taken up at high amounts, causes oxidative stress in plants (Vitoria et al., 2001; Dixit et al., 2001). It is well documented that increase in oxidative stress conditions lead to increased production of reactive O_2 species (ROS) (Bowler et al., 1992; Foyer et al., 1997). In plants, reactive O_2 species are synthesised especially during the photosynthetic electron transport in chloroplasts. Under stress conditions, like heavy metal stress, the photosynthetic electrons cannot be used in the reduction of CO_2; therefore, they accumulate in chloroplasts and are then transferred to molecular O_2 with concomitant activation of O_2. Because of O_2 activation, highly reactive O_2 species ($O_2^{\cdot -}$, superoxide radical; H_2O_2, hydrogen peroxide; $OH^{\cdot}$, hydroxyl radical and $^1O_2$, singlet oxygen) are produced (Asada, 1994, 2000; Foyer et al., 1994; Cakmak, 1994, 2000). The toxic O_2 species exert strong oxidizing effects on proteins, membrane lipids, chlorophyll, DNA and other cellular components leading to cell death.

Plants developed different enzymatic and non-enzymatic defence mechanisms against oxidative stress induced by ROS. Among the enzymatic reactions, superoxide dismutase (SOD) is involved in detoxification of $O_2^{\cdot -}$, and catalase, ascorbate peroxidase and glutathione reductase are involved in detoxification of H_2O_2 (Asada, 1992; Foyer et al., 1994). Antioxidants like ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, β-carotene and zeaxanthin carotenoids are the most important antioxidative defence metabolites of plants against ROS (Cakmak and Marschner, 1992; Demming-Adams and Adams, 1996; Noctor and Foyer, 1998; Foyer and Noctor, 2000).

Among plant species and even genotypes of given species there is great genotypic variation in Cd tolerance (Prasad, 1995; Grant et al., 1998). The reason for such large

genotypical differences is still not well understood. Plants developed several tolerance mechanisms against Cd toxicity such as restricted Cd influx through plasma membrane, exclusion of Cd (active pumping of Cd out of the cell), compartmentalisation of Cd at the cellular level, detoxification of Cd by Cd-binding peptides or proteins (Grant et al., 1998; di Toppi and Gabbrielli, 1999; Cobbett, 2000). Plants can also tolerate Cd toxicity by inducing antioxidative defence system. As mentioned above, Cd stress can be responsible for production of toxic O_2 species and peroxidation of critical cell compounds, such as membrane lipids and proteins, chlorophyll and nucleic acids (Chaoui et al., 1997; Dixit et al., 2001; Vitoria et al., 2001; Shah et al., 2001; Hegedüs et al., 2001). An induced antioxidative defence in response to Cd stress might be, therefore, a relevant mechanism for Cd tolerance in plants.

Although there are large numbers of studies dealing with the relationship between Cd toxicity and generation and detoxification of ROS in plants, very little attention was, paid to the role of antioxidative defence systems in genotypic variation against Cd toxicity (Shah et al., 2001). Therefore, in this MSc thesis project, experiments were conducted to assess the role of antioxidative defence system in Cd tolerance between two barley cultivars. Experiments were realised under controlled environmental conditions using Tokak (Cd-tolerant) and Hamidiye (Cd-sensitive) barley cultivars by giving attention to the uptake and accumulation of Cd, dry matter production, development of Cd toxicity symptoms and levels of antioxidative defence systems.

2. PREVIOUS STUDIES

2.1. Cadmium in Soil

There are several factors increasing Cd concentration up to toxic levels in soil such as soil chemical characteristics, agricultural, manufacturing, mining and other waste disposal practices and use of metal containing pesticides and application of fertilisers to agricultural soils (Smith et al., 1996; Grant et al., 1998; Shallari et al., 1998). Average Cd concentration of the earth is thought to be around 0.1 mg kg^{-1} (Bowen, 1979). Concentrations of Cd in soil solution have been reported to vary from $9x10^{-9}$ M to $1.6x10^{-9}$ M (Keller, 1995). Precipitation, transpiration, humidity, pH and soil permeability are major factors affecting the rate and intensity of Cd transport in soils (Kabata and Pendias, 1992). Soil factors affecting Cd uptake by plants include the concentration of Cd in soil solution, soil pH, cation exchange capacity, redox conditions and concentrations of other heavy metals. Uptake of Cd by plants increases with the increasing concentration of Cd in soil, and is influenced by the size and uptake characteristics of the plant root system (Hart et al., 1998; Grant et al., 1998). The pH of the soil exerts a major influence on Cd availability by affecting its absorption by plant roots and its chemical forms in soil. Cadmium uptake is inversely related with pH: increasing pH causes a reduction in uptake of Cd. Cation exchange capacity and redox condition also play a role in the availability of Cd to plant roots. While some research has shown that Cd uptake and cation exchange capacity exhibited a negative relation, others stated that there was not a meaningful correlation (Alloway, 1995; Grant et al., 1998; Moral et al., 2002). Furthermore, cadmium-zinc

interaction is an important factor affecting Cd uptake from soil and Cd transport to plant roots (Welch et al., 1999; Cakmak et al., 2000a). Zinc and Cd are chemically similar and compete for the binding sites in the soil system and for the uptake sites in cell membranes (Christensen, 1984; Abdel-Sabour et al., 1988; Cakmak et al., 2000a).

2.2. Cadmium in Plants

Plant species greatly differ in their ability to absorb, accumulate and tolerate heavy metals (Patterson et al., 1977; Guo et al., 1995; Hart et al., 1998; Grant et al., 1998). For example, maize, beans and peas accumulate Cd in very small amounts whereas cucumber, spinach, celery and cabbage accumulate Cd in very high amounts. Cadmium distribution within plants is another parameter that affects Cd absorption and accumulation. Some plant species transport Cd preferentially to shoots more readily while others accumulate Cd in roots (Wagner, 1993; Grant et al., 1998).

In fact, all heavy metals like Cd, Mn, Zn, B, Mo and Se, after taken up by plant roots, are easily transported to shoots. Among the heavy metals Cd is of particular interest because it is known to be taken up more readily by the roots of many plant species and tends to be 2 to 20 more times toxic than other heavy metals (Jagodin et al., 1995; Das et al., 1997). In addition, mobility of Cd in soils is much higher than other heavy metals, and therefore the transport of Cd in the soil-water systems seems to be a fast process (Moral, 2002).

The mechanisms for Cd uptake, translocation and compartmentalisation are not yet well understood, but in the past years, an important effort has been made in this direction (Grant et al., 1998; di Toppi and Gabbrielli, 1999; Cobbett, 2000). As indicated above, plant species as well as genotypes of a given plant species differ in absorbing, accumulating

and tolerating heavy metals (Florjin and Van Beusichem, 1993; Guo et al., 1995; Hart et al., 1998; Cakmak et al., 2000b). McLaughlin and his co-workers (1994) showed that 14 potato cultivars growing in South Australia contained different levels of Cd. The variation in Cd concentration in potatoes cultivars was between 30 to 50 $\mu g\ kg^{-1}$ (in fresh weight). Similar genotypic variation in Cd uptake and accumulation was also found by Boges et al. (1978) in soybean and corn, John and von Laerhoven (1976) in cucumber, Petterson (1977) in wheat and barley and John and Van Laerhoven, (1976) in lettuce. These results indicate that development of Cd-tolerant genotypes is possible by exploiting existing genetic variation.

The translocation of Cd from roots into shoots is an important factor involved in expression of genotypic differences in Cd tolerance, for example in tomato (Moral, 2002) and peanuts (McLaughlin et al., 2000). Metal translocation from roots to shoots is a long distance transport controlled by several physiological processes including metal unloading into root xylem cells, long distance transport within the xylem to the shoots and metal reabsorption from the xylem stream by leaf mesophyll cells (Raskin and Ensley, 2000). Since the roots are the first barrier system of plants to heavy metals in the soil (Vassilev, 1998; Grant et al., 1998), Cd-treatments mainly affect roots, and Cd taken up by plants mostly accumulate in roots (Hegedus et al., 2001; Vitoria et al., 2001). In grasses, 65-90% of the total Cd is located in the roots (Davies, 1980). However, in lettuce, only 50% of the total Cd is found in the roots (Ramos et al., 2002).

Accumulation of Cd in shoots or roots at excessive amounts leads to severe damages to plant cells. Cadmium toxicity results in negative effects on photosynthetic processes in plants by blocking activities of several photosynthetic enzymes, particularly the enzymes involved in the Calvin cycle and chlorophyll biosynthesis (Van Assche and Clijsters, 1990; Krupa et al., 1993; Chug and Sawhney, 1999). Typical visible symptoms of Cd toxicity include development of reddish-brown necrosis and chlorosis on leaves, curled leaves, browning of roots and general reduction in root and shoot growth (Ouzounidou et al., 1997). In barley plants, Cd toxicity was found to be associated with increasing

chlorophyll degradation and lipid peroxidation. These effects were associated by increased ascorbate peroxidase, an enzyme scavenging H_2O_2 (Hegedüs et al., 2001). Increases in activity of ascorbate peroxidase indicate Cd-induced H_2O_2 production in plant cells. Vitoria et al. (2001) and Dixit et al. (2001) reported that besides ascorbate peroxidase, also glutathione reductase showed an increase under Cd toxicity. As discussed in more detail below, glutathione reductase together with ascorbate peroxidase is involved in H_2O_2 detoxification during ascorbate-glutathione cycle (Foyer et al., 1994; 1997; Noctor and Foyer, 2000).

2.3. Generation and Detoxification of ROS in Plants

Production of ROS is enhanced by various environmental stress factors including heat, high light, drought, mineral nutrient deficiency, heavy metals, extreme temperatures and UV radiation (Bowler et al., 1992; Simirnoff et al., 1993; Foyer et al., 1994, 1997; Scandalios et al., 1997). Ability of Cd to produce ROS in plants was reported by Assche and Clijsters (1990) who observed enhanced expression of new isoenzymes of peroxidase in both roots and leaves of *Phaseolus vulgaris*. Other evidence of Cd inducing oxidative stress came from the results showing increases in lipid peroxidation and chlorophyll breakdown under Cd toxicity (Somashekaraiah et al., 1992; Gallego et al., 1996; Chaoui et al., 1997; Dalurzo et al., 1997).

The reactive O_2 species are highly toxic and cause severe damage in plants in different ways. They rapidly destroy chlorophyll and membrane lipids and inactivate proteins by oxidizing SH-groups. These effects result in photooxidative damage in chloroplast activity, especially under high light intensity (Foyer et al., 1994; Gressel and Galun, 1994; Foyer et al., 1997).

Understanding the biochemical detoxification strategies of plants against ROS is important. Unless ROS are detoxified, they cause various damages and lead to plant death. Development of adaptive mechanisms by plants against oxidative stress induced by accumulation of metals play a crucial role in expression of heavy metal tolerance in plants. There is increasing evidence showing that exposure of plants to Cd activates various tolerance mechanisms such as complexing of metal by Cd-binding peptides or proteins (i.e., phytochelatins and metallothioneins), vacuolar compartmentalization, immobilization at the level of cell wall, preventing uptake of Cd into cytosol (exclusion) and synthesis of stress proteins and antioxidative defense mechanisms (Grant et al., 1998; Sanita di Toppi and Gabrielli, 1999; Cobbett, 2000; Clemens, 2001). Antioxidative mechanisms consist of antioxidants and enzymes, which either are directly involved in the removal of toxic oxygen species or are necessary for the generation of the reduced state of antioxidative substrates. The antioxidants, ascorbate (vitamin C), glutathione and α-tocopherol (vitamin E) participate as substrates in enzymatic reactions or directly scavenge toxic radicals in non-enzymatic reactions (Asada and Takahashi, 1987; Foyer et al., 1997; Foyer and Noctor, 2000).

In enzymatic defence mechanisms of plants against ROS, superoxide dismutase (SOD), ascorbate peroxidase (AP), glutathione reductase (GR) and catalase play an important role. These enzymes can be key elements in alleviation Cd-induced oxidative stress in plants. Superoxide dismutase (SOD) catalyses dismutation of $O_2^{.-}$ to H_2O_2 and O_2 (Bowler et al., 1992; Scandalios, 1993). Thus, SOD maintains a low steady state concentration of superoxide radical and, therefore, minimises hydroxyl radical formation by $O_2^{.-}$ catalysed Haber-Weiss reaction (Elstner, 1982; Bowler et al., 1992). Hydrogen peroxide is broken down by catalase and peroxidases (Asada, 1992; Scandalios, 1994). Catalases scavenge H_2O_2 generated during the photorespiration and β-oxidation of fatty acids (Elstner, 1982; Elstner et al., 1988). The protective action of catalase against H_2O_2 is limited because of its low affinity to H_2O_2 and high sensitivity to light induced inactivation, and it is only localised in peroxisomes (Elstner, 1987; Foyer et al., 1994). Hydrogen peroxide is directly involved in oxidation of SH- containing enzymes of the Calvin Cycle

and thereby inhibition of photosynthesis (Tanaka et al., 1982). Hydrogen peroxide is also responsible for the production of the potent oxidant $OH^{\cdot}$ via Fenton reaction. When compared to catalase, in plant cells, an alternative and more effective H_2O_2-scavenging mechanism exists, operating both in chloroplast and the cytosol, which is called “ascorbate-glutathione pathway” (Fig. 2.1). As described by Asada and Takahashi (1987) and Sharma and Davies (1997), in this detoxification mechanism H_2O_2 is reduced to H_2O by ascorbate peroxidase (AP). For the reduction of H_2O_2 to H_2O, ascorbic acid is used as an electron donor, and during this reaction MDA (monodehydroascorbate) and DHA (dehydroascorbate) are formed. The regeneration of reduced ascorbate (AsA) from MDA or DHA, which are the reaction products of AP, can be catalysed either by NADH-dependent MDAsA reductase or by GSH-dependent DHAsA reductase, coupled with glutathione reductase (GR) (Fig. 2.1) With the participation of dehydroascorbate (DHA), glutathione (GSSG) that was oxidised during the regeneration of ascorbic acid is again converted to the reduced form (GSH) by the activity of GR. During this reaction, NADPH is used as an electron donor (Fig. 2.1) (Cakmak, 1994; Foyer et al., 1994, 1997; Sharma and Davies, 1997).

In chloroplasts $O_2^{\cdot -}$ (superoxide radical) is produced by the transfer of photosynthetic electrons to molecular O_2. It is estimated that under normal conditions about 10-20 % of the released electrons during photosynthetic electron transport are used for reduction of O_2 to $O_2^{\cdot -}$ (Asada et al., 1977; Robinson, 1988; Osmond and Grace, 1995; Cakmak and Engels, 1999). Generation of $O_2^{\cdot -}$ in chloroplasts is intensified when plants exposed to environmental stress conditions like chilling, high light and high temperature, drought and heavy metal stress (Elstner et al., 1988; Polle, 1996; Foyer et al., 1997; Cakmak, 2000). These stress conditions limit photosynthetic CO_2 fixation, thus intensify electron flow to O_2 instead of CO_2 and consequently production of $O_2^{\cdot -}$ and $O_2^{\cdot -}$ -derived other toxic ROS such as H_2O_2 and $OH^{\cdot}$. It appears that under stress conditions, e.g., Cd stress, activity of $O_2^{\cdot -}$ -scavenging SOD and H_2O_2 -scavenging ascorbate-glutathione pathway (Fig. 2.1) play a crucial role in protection of cells from damaging attack of ROS.

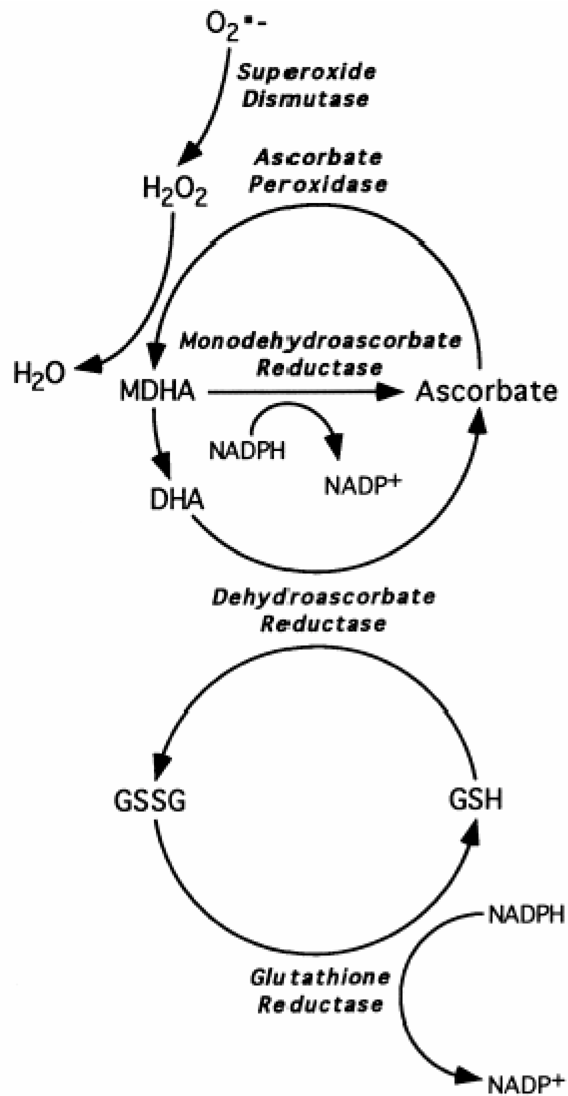

Fig. 2.1 The ascorbate-glutathione pathway operating mainly in chloroplasts to detoxify H_2O_2. (from: Sharma and Davies, 1997). MDHA: monodehydroascorbate, DHA: dehydroascorbate, GSSG: oxidised glutathione and GSH: reduced glutathione.

Ascorbic acid, vitamin E, glutathione and carotenoids are well-described antioxidants which plants use to detoxify the toxic O_2 radicals formed under various stress conditions (Cakmak and Marschner, 1992; Demming-Adams and Adams, 1996; Noctor and Foyer, 1998; Conklin, 2001). In plant cells, ascorbic acid is the most important substrate in the ascorbate-glutathione pathway (Fig. 2.1). Ascorbic acid has the ability to reduce the superoxide, hydrogen peroxide and hydroxyl radicals and to react directly with singlet oxygen (Foyer et al., 1997; Noctor and Foyer, 1998; Conklin, 2001). Ascorbic acid is found in chloroplasts, cytosol and vacuole and in the apoplastic spaces of the leaf cells in high concentrations (Foyer et al., 1994; Badiani et al., 1996). The other member of the ascorbate-glutathione cycle is glutathione. It plays a role in the regeneration of ascorbic acid by dehydroascorbate reductase (Fig. 2.1). Glutathione can react directly with singlet oxygen ($^1O_2$) and hydroxy radical ($OH^.$) and thus prevents SH-groups of enzymes from oxidising attacks of $^1O_2$ and $OH^.$. Glutathione is also needed for synthesis of Cd-binding peptides, phytochelatins (Cobbett, 2000; Clemens, 2001). When taken up by plants, Cd is able to activate synthesis of phytochelatins by stimulating conversion of glutathione to phytochelatins. These phytochelatins are known to bind and complex Cd and play an important role in vacuolar compartmentalisation of Cd and thus expression of Cd tolerance in plants (Cobbett, 2000; Clemens, 2001).

In this MSc work, nutrient solution experiments were carried out under controlled environmental conditions to study the role of Cd accumulation and antioxidative defence mechanisms in differential expression of Cd tolerance between two barley cultivars, Tokak and Hamidiye. In preliminary experiments we found that Tokak (Cd-tolerant) and Hamidiye (Cd-resistant) are markedly different in their tolerance to Cd toxicity as judged from the occurrence of leaf symptoms and growth retardation due to Cd toxicity.

3. MATERIALS AND METHODS

3.1. Materials

Two cultivars of barley (Hamidiye and Tokak) were used in the experiments. After surface sterilization with 1% (w/v) calcium hypochlorite for 20 min, seeds were sown in perlite and kept in the dark for approximately 4 days. Afterwards, the seedlings were transferred to 2.5 L plastic pots containing continuously aerated nutrient solution. Nutrient solution had the following composition: 2 mM $Ca(NO_3)_2.4H_2O$, 1 mM $MgSO_4.7H_2O$, 0.9 mM K_2SO_4, 0.2 mM KH_2PO_4, 10^{-6} M H_3BO_3, $2x10^{-7}$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 $C_{10}H_{12}FeN_2NaO_8$.

Plants were grown in growth chambers under controlled environmental conditions (temperature 20°C in the light and 18°C in the dark, 16-h light/8-h dark photoperiod with a photon flux density of 380 µmol m^{-2} s^{-1} and 60 % humidity). When plants were grown for 3 days in nutrient solution after transfer from perlite, Cd was added in the solution at different concentrations as indicated in the legends of Tables and Figures. Cadmium was supplied in the form of $3CdSO_4.8H_2O$. Following Cd applications plants were harvested at different time intervals. At harvest, roots were rinsed with $CaSO_4$ and deionised water, and the dried root and shoot samples were then ground and digested in a microwave for measurement of Cd. Measurements of antioxidative enzyme activities, antioxidants and lipid peroxidation were carried out on leaf samples. The harvested leaf samples were

treated with liquid N and then directly used for analysis or stored at -20^0C until next analysis. As the leaves supplied with 120 µM Cd were highly damaged, they were not used for enzyme determination.

3.2. Methods

3.2.1. Dry Weight and Cadmium Tolerance Index

Plants dried at 70^oC 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 supplies to that without Cd supply (control treatment) as following:

Cd Tolerance Index: Dry weight at Cd supply/dry weight at nil Cd supply x 100.

3.2.2. Cadmium Concentration and Content

Approximately 0.5 g ground plant samples were digested in a microwave using 2 ml H_2O_2 and 4 ml HNO_3. The digested samples were analysed for Cd by using inductively coupled plasma (ICP) atomic emission spectrometer (ICP-AES) (Jobin Yvon- France). Measurement of Cd was checked using certified Cd values in reference leaf materials obtained from the National Institute of Standards and Technology (Gaithersburg, USA). The Cd content was calculated by multiplying the dry weight of root or shoot with their Cd concentration.

3.2.3. Determination of Lipid Peroxidation

Lipid peroxidation was measured as described by Hodges et al. (1999). Approximately 0.5g fresh leaf example was homogenised in 80 % ethyl alcohol and centrifuged at 3000 rpm. After centrifuging, the extract obtained was analysed in two steps. At the first step, 1 volume of 20 % (mass/volume) TCA (trichloroacetic acid) and 1 volume of 0.01 % BHT (buthylated hydroxytoluene, an antioxidant used to block lipid peroxidation during the assay) were added to 1 volume of supernatant. At the second step, 1 volume of 20 % TCA that contained 1 volume of 0.65 % TBA (2-thiobarbituric acid) and 1 volume 0.01 % BHT were added to 1 volume extract taken from the supernatant. After vortexing the examples for 10 seconds, they were incubated in a hot water bath adjusted to 95°C for 25 minutes followed immediately by a shock treatment in an ice bath. The cooled samples were centrifuged at 3000 rpm, and absorbance values of supernatants were measured in spectrophotometer. First step samples were measured at 532 and 600 nm, whereas second step samples at 400, 532, and 600 nm. Results were obtained using the following formulas (ABS: Absorbance, MDA: Malondialdehid).

$$[(\text{ABS } 532_{+TBA}) - (\text{ABS } 600_{+TBA}) - (\text{ABS } 532_{-TBA}) - (\text{ABS } 600_{-TBA})] = A$$

$$[(\text{ABS } 440_{+TBA} - \text{ABS } 600_{+TBA}) \text{ x } 0.0571] = B$$

$$\text{nmol MDA / ml} = (\text{A-B / } 157000) \text{ x } 10^{6}$$

3.2.4. Determination of Soluble Protein Contents

Protein content was determined using bovine serum albumin as a standard as described in Bradford (1976). The protein assay reagent was prepared as follows: 100 mg

coomassie brillant blue G 250 is 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 filtered. Right after filtration, 100 ml of glycerol (about 87 %) is added and filled up to 1000 ml with deionised water. This reagent was used in protein measurements in the enzyme extracts. For measurement of soluble protein, sample solution of 100 μL (enzyme extract) and 5 ml of protein assay reagent were mixed together. After vortexing the reagent-sample mixture, the colour produced was measured at 595 nm versus reagent blank. The bovine serum albumin standards were prepared in the range of 0 to 1000 μg ml^{-1}.

3.2.5. Determination of Non-Protein SH-Groups

Soluble non-protein groups were determined using 5-5'-dithiobis- (2-nitro benzoic acid) (DTNB) under 5 % *meta*-phosphoric acid as a reagent as described in Cakmak and Marschner (1992). Accordingly, 0.5g plant sample was homogenised in 5 % *meta*-phosphoric acid and centrifuged at 4000 rpm. The reaction mixture contained 0.5 ml aliquot of the supernatant, 2.5 ml 150 mM phosphate buffer (pH 7.4) containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 ml 6mM 5-5'-dithiobis- (2-nitrobenzoic acid). After incubation at room temperature for 20 min reaction time, the colour produced was measured at 412 nm using reduced glutathione as a standard in the range of 0 to 100 μg ml^{-1}.

3.2.6. Determination of Total Ascorbate Levels

Total ascorbate was determined according to Cakmak and Marschner (1992) with some modifications. Approximately 0.5 g leaf samples were extracted with 5 ml of 5 % *meta*-phosphoric acid, and centrifuged at 4000 rpm for 30 min. Total ascorbate

(AsA+DHAsA) was measured after reduction of DHAsA to AsA with DTT (1,4 dithiothreitol). The reaction mixture contained 0.2 ml aliquot of the 4000 rpm supernatant, 0.5 ml 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA, 0.1 ml 10 mM DTT and 0.1 ml 0.5 % (w/v) *N*-ethylmaleimide (NEM) to remove excess DTT. In the reaction mixture, the colour was developed after addition of the following reagents: 0.4 ml 10 % trichloroacetic acid (TCA), 0.4 ml 44 % *ortho*-phosphoric acid, 0.4 ml 4 % 2,2'-bipyridine in 70 % ethyl alcohol, and 0.2 ml 3 % $FeCl_3$. The mixtures were then incubated at 40°C for 40 min, and the colour produced was measured at 525 nm. L(+)ascorbic acid was used as a standard in the range of 0 to 100 µg ml^{-1}.

3.2.7. Assays of Antioxidative Enzymes

Approximately 0.5g fresh leaf samples were homogenised in 50mM phosphate buffer (pH 7.6) including 0.1 mM Na-EDTA. Samples were generally homogenized in 8 ml, and then centrifuged for 15 minutes at 20 000 rpm and 4°C.

3.2.7.1. Ascorbate Peroxidase (AP)

Activity of ascorbate peroxidase (AP) was measured according to Cakmak (1994) by monitoring the rate of H_2O_2-dependent oxidation of ascorbate at 290 nm (E=2.8 mM cm^{-1}). The reaction mixture (1 ml) contained 50 mM phosphate buffer (pH 7.6), 0.1 mM EDTA, 12 mM H_2O_2, 0.25 mM ascorbic acid and the enzyme aliquot.

3.2.7.2. Glutathione Reductase (GR)

Glutathione reductase was assayed using the method described by Cakmak and Marschner (1992) and Cakmak (1994) by following the decrease in absorbance at 340 nm due to NADPH oxidation (E=6.2 mM cm^{-1}). The reaction mixture (1 ml) contained 50

mM phosphate buffer (pH 7.6), 0.1 mM EDTA, 0.5 mM oxidised glutathione (GSSG), 0.12 mM NADPH (0.1 ml), and the enzyme aliquot. Corrections were made for NADPH oxidation in the absence of GSSG.

3.2.7.3. Superoxide Dismutase (SOD)

Superoxide dismutase activity was assayed using the method of Cakmak and Marschner (1992), and based on the inhibition of nitro blue tetrazolium chloride reduction by $O_2^{\cdot -}$ under light intensity. For the assay of SOD, the reaction medium (5 ml) containing 50 mM phosphate buffer (pH 7.6), 0.1 mM Na-EDTA, enzyme aliquots (50-150 µl), 50 mM Na_2CO_3 (pH 10.2), 12 mM L-methionine, 75 µM *p*-nitro blue tetrazolium chloride (NBT) and 2 µM riboflavin was maintained in glass vials. Riboflavin was the last compound to be added. Reactions were carried out at room temperature and under a light intensity of about 400 µmol m^{-2} s^{-1} for 10 min. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the rate of NBT reduction measured at 560 nm.

3.2.7.4. Catalase

Catalase activity was measured as described by Cakmak and Marschner (1992). The assay was based on the degradation of H_2O_2 at 240 nm (E=39.4 mM cm^{-1}), and the reaction medium (1 ml) contained 50 mM phosphate buffer (pH 7.6), 0.1 mM EDTA, 0.1 ml 100 mM H_2O_2 and the enzyme aliquot. The decrease in H_2O_2 was monitored at 240 nm.

For statistical treatments see legends of Tables and figures.

4. RESULTS

4.1. Leaf Symptoms

The first reaction of both barley cultivars to increasing Cd supply was the reduction in shoot length (Fig. 4.1). Associated with this observation, leaves showed development of reddish-brown patches on the older leaves, especially along the leaf margins. Younger leaves did not show reddish-brown patches, but had a reduced size due to Cd toxicity. Although both cultivars seemed to respond similarly to increasing Cd supply regarding the decrease in shoot length, but they differed greatly in development time and severity of reddish-necrotic patches (Figs. 4.1 and 4.2). Hamidiye was highly affected by Cd toxicity, and developed very rapidly reddish-necrotic patches on leaves, and with time the leaves of Hamidiye totally collapsed (Figs. 4.2 and 4.3). In the case of Tokak, the reddish-necrotic patches were less obvious on the leaves. For example, at a Cd supply of 15µM Hamidiye showed leaf symptoms of Cd toxicity already within 24 h, while in Tokak leaf symptoms occurred first after 48 h and only at very high Cd supplies (e.g., 60 or 120 µM Cd).

A

B

Fig. 4.1 Growth of the barley cultivars Hamidiye (A) and Tokak (B) with increasing Cd supply in nutrient solution for 48 hours. Plants were grown until 7 days without Cd, and then supplied with increasing application of Cd. Cadmium treatments from left to right: control (0 μM), 15μM, 30 μM, 60 μM and 120 μM $CdSO_4$ was applied.

A

B

Fig. 4.2 Leaf symptoms of Cd toxicity on Hamidiye (A) and Tokak (B) grown in nutrient solution with 120 µM Cd supply for 48 h. The plants were 7 days-old before the Cd supply.

Fig. 4.3 Differences in growth and severity of leaf symptoms of barley cultivars Tokak (left) and Hamidiye (right) under 120 μM Cd supply for 48 hours. Plants were grown until 7 days without Cd supply and then Cd was applied.

4.2. Growth and Cadmium Tolerance Index

Both cultivars were severely affected by Cd, and showed a continuous decrease in the dry weight of shoots and roots with Cd treatments (Table 4.1). With increasing Cd supply from 0 to 120 μM the shoot dry weight of Hamidiye decreased by 40 %, while the decrease in Tokak was around 30 %. It seems that the decrease in shoot dry weight with Cd is similar between Tokak and Hamidiye at low Cd supply, but become greater in Hamidiye at higher Cd supplies. At the low supply of Cd (e.g., 15μ M) shoot dry weight decreased by 31 % in Hamidiye and 29 % in Tokak (Table 4.1).

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 nearly same at 15 µM Cd supply, but showed a considerable difference between the cultivars at higher Cd supplies, being greater in Tokak than Hamidiye (Table 4.1). This indicates again greater Cd tolerance of Tokak than Hamidiye.

Also in the case of root dry weight, Hamidiye was more sensitive to Cd supply than Tokak. Root dry weight of Hamidiye decreased by 40 % at 15 µM Cd, 42 % at 30 µM Cd, 49 % at 60 µM Cd and 53 % at 120 µM Cd while these decreases in root dry weight for Tokak were 24 %, 20 %, 36 % and 40 %, respectively (Table 4.1). Accordingly, the Cd tolerance index for root growth was lower in Hamidiye than Tokak at all Cd concentrations (Table 4.1).

Table 4.1 Shoot and root dry weight and the tolerance index of the 9 days-old barley cultivars Hamidiye and Tokak which were grown for 48 h by increasing Cd concentrations in nutrient solution before the harvest. Data represent means ± SD of three independent experiments.

Cd supply	Dry Weight		Tolerance index	
	Hamidiye	Tokak	Hamidiye	Tokak
(µM)	(mg plant^{-1})		(%)	
	Shoot			
0	1293±60	1134±71		
15	893±38	801±30	69.0	70.6
30	903±67	893±6	69.9	78.7
60	833±42	800±26	64.4	70.5
120	774±12	777±35	59.8	68.5
	Root			
0	573±33	504±42		
15	340±17	377±15	59.3	74.8
30	327±15	403±6	57.0	79.9
60	293±23	323±15	51.1	64.0
120	273±12	297±25	47.6	58.9

In the experiment related to the time-response of the cultivars to the 60 μM Cd supply, both cultivars were affected by Cd already following 24 h treatment. The decreases in shoot and root dry weights were proportional with the duration of the Cd treatment, and similar between Hamidiye and Tokak (Table 4.2). Following 36h treatment of Cd Hamidiye was more severely affected than Tokak. The leaf symptoms of Cd toxicity occurred first in Hamidiye following 24 h Cd supply. Also in this experiment, the older leaves of Hamidiye showed necrotic patches, pronounced around leaf margins within 24 h of Cd treatment, while in Tokak the visual symptoms of Cd toxicity were observed following 48 h or 60 h of Cd supply. At 36 h of Cd application, tolerance index for shoot growth was 82.9 % in Hamidiye and 92.9 % in Tokak. Following 60 h of Cd treatment Cd tolerance index for shoot growth was more or less similar between Hamidiye and Tokak (Table 4.2).

When compared to the shoot growth, the root growth was much more sensitive to Cd supply, especially at higher doses and longer application period of Cd (Tables 4.1 and 4.2). In roots of Hamidiye, the decrease in root dry matter production was only 2 % after 12 h of Cd supply when compared to the control plants. With increasing application period of Cd decreases in root dry weight were calculated to be 4 % for 24 h, 26 % for 36 h, 36 % for 48 h and 41 % for 60 h. In the case of Tokak, no loss in dry matter production was detected following 12 h Cd supply. Decreases in root growth with increasing duration of Cd supply were very similar to those obtained with Hamidiye (Table 4.2).

Table 4.2 The effect of increasing time of Cd 60 µM Cd supply on the shoot and root dry weight and the tolerance index of Hamidiye and Tokak cultivars grown 9 days in nutrient solution. Cadmium tolerance index was calculated as the ratio of dry weight at Cd supply to the dry weight at control (no Cd) treatment. The results show the means (± SD) of three independent experiments.

Cultivars	Application period (h)	Cd 0 (mg DW plant^{-1})	Cd 60 (mg DW plant^{-1})	Tolerance index (%)
		Shoot		
Hamidiye	12	463±29	460±12	99,3
	24	434±55	396±33	91,2
	36	616±40	511±15	82,9
	48	635±13	537±35	84,6
	60	858±42	619±29	72,1
Tokak	12	416±22	415±8	99,8
	24	402±7	369±7	91,8
	36	537±55	499±30	92,9
	48	557±22	485±11	87,1
	60	815±49	590±15	72,4
		Root		
Hamidiye	12	166±4	169±10	100
	24	185±13	177±20	95,7
	36	245±3	182±7	74,3
	48	273±7	176±8	64,5
	60	304±6	180±5	59,2
Tokak	12	156±10	156±10	100
	24	177±12	163±7	92,1
	36	216±15	172±4	79,6
	48	254±9	180±14	70,9
	60	326±27	175±13	53,7

4.3. Cadmium Concentration and Content

Irrespective of the cultivars, a close relationship was observed between the Cd supply and the Cd concentrations of plants (Table 4.3). Increasing Cd supply enhanced Cd concentration in shoots and particularly in roots. Most of the Cd taken up by the plant accumulated in the roots and an only small amount of Cd was translocated to the leaves. Hamidiye tended to take up and accumulate more Cd than Tokak. At 15 μM Cd supply, Cd concentrations of shoots were 78 mg kg^{-1} DW in Hamidiye and 57 mg kg^{-1} DW in Tokak. The Cd concentrations of Hamidiye also remained to be higher in other Cd treatments compared to Tokak. Generally, Hamidiye had 13 % more Cd in shoots compared to Tokak at all Cd doses. The content of Cd (the total amount of Cd per shoot) was also higher in Hamidiye than (Table 4.3). With increases in Cd supply from 0 to 120 μM, Hamidiye contained 27 %, 24 %, 22 % and 20 % more Cd in shoot when compared to Tokak, respectively (Table 4.3). Similar differences were also obtained for roots. Cadmium concentrations of roots varied between 0.8 to 3743 mg kg^{-1} DW in Hamidiye, and 0.22 to 3164 mg kg^{-1} DW in Tokak (Table 4.3).

Table 4.3 Effect of increasing Cd supply on concentration and content of Cd in shoot and roots of 9 days-old barley cultivars Hamidiye and Tokak. Plants were exposed to 48 h of Cd supply before the harvest. The results show the means (± SD) of three independent experiments.

Cd Supply	Cd concentration		Cd Content	
	Hamidiye	Tokak	Hamidiye	Tokak
(µM)	(mg kg^{-1} DW)		(µg $plant^{-1}$)	
		Shoot		
0	0.096±0.04	0.034±0.02	0.124±0.56	0.038±0.23
15	78±3.1	57±5.7	69.9±4.00	46.1±6.19
30	91±3.0	69±2.0	82.0±8.18	61.6±1.53
60	117±15	91±1.0	99.6±17.3	73.1±1.99
120	138±13	110±4.0	106.3±8.77	85.7±1.24
		Root		
0	0.83±0.2	0.22±7	0.471±0.13	0.11±3.34
15	1480±120	1212±84	501.9±18.8	456.7±43.5
30	2138±47	1732±40	698.0±17.4	698.7±17.4
60	2418±52	2129±183	710.0±71.2	688.8±71.6
120	3743±185	3164±111	1022.2±37.5	938.0±75.2

4.4. Cadmium Uptake

Both barley cultivars seemed to be different in uptake capacity for Cd. On average, Hamidiye tended to accumulate more Cd in shoots, but less Cd in its roots in comparison to Tokak (Table 4.4). At the 12th h period of 60 µM Cd treatments, no variation was detected in the uptake of Cd in shoots between two cultivars. However, at the other time periods there were variations among the cultivars in the uptake and translocation of Cd. The highest variation in uptake of Cd was found at the 60th h of Cd treatment. At this time, Hamidiye had 22 % higher Cd in shoots when compared to Tokak.

Interestingly, most of Cd found in shoot was accumulated within 24 h (Table 4.4). At the beginning of Cd exposure, there was a rapid Cd uptake; then, the Cd concentration and content of plants were slowed with the time of Cd exposure (Table 4.4).

Table 4.4 Influence of exposure time of 60 µM Cd on concentration and content of Cd in root and shoots of 9 days-old barley cultivars grown in nutrient solution. The results show the means (± SD) of three independent experiments.

Cultivars	Application period (h)	Cd concentration (mg kg^{-1})	Cd Content (µg plant^{-1})
		Shoot	
Hamidiye	12	37±5.3	16.9±2.7
	24	102±8.5	40.3±0.9
	36	114±1.4	58.0±0.9
	48	131±5.7	70.6±1.5
	60	138±2.1	75.0±2.7
Tokak	12	37±3.5	15.5±1.5
	24	86±2.9	22.5±1.7
	36	100±0.8	50.0±2.4
	48	122±9.6	59.2±3.9
	60	107±3.3	63.3±4.8
		Root	
Hamidiye	12	656±109	111.6±24.4
	24	750±150	130.6±12.6
	36	1010±46	183.9±12.9
	48	1236±36	216.7±4.4
	60	1626±270	291.5±29.9
Tokak	12	682±36	106.1±7.8
	24	785±22	127.8±9.1
	36	1133±100	195.2±20.1
	48	1283±62	231.0±11.6
	60	1766±6	318.1±8.8

When the same experiment shown in Table 4 was repeated with a much lower Cd supply (i.e., 5 μM) similar results were found again (Table 4.5). This result indicates that the concentration of Cd applied in the nutrient solution does not play an important role in Cd uptake and accumulation between Tokak and Hamidiye. Greater uptake and accumulation of Cd at earlier exposure time of Cd were found also in this experiment. Especially in the case of Cd content, most of Cd in shoot and root was found following 24 h exposure to Cd (Table 4.5). This result suggests that uptake and accumulation of Cd are not proportional to growth rate of plants.

Table 4.5 Influence of exposure time of 5 μM Cd on concentration and content of Cd in root and shoots of 9 days-old barley cultivars grown in nutrient solution. The results show the means (± SD) of three independent experiments.

Cultivars	Application Period (h)	Cd concentration (mg kg^{-1})	Cd Content (μg $plant^{-1}$)
		Shoot	
Hamidiye	12	9.0±0.62	5.7±0.6
	24	23.3±2.88	14.0±2.5
	36	31.8±1.83	24.0±1.4
	48	41.7±2.52	32.1±2.4
	60	34.2±1.38	33.6±1.7
Tokak	12	8.4±1.00	5.00±0.8
	24	19.8±1.59	11.2±1.2
	36	26.5±2.73	20.1±2.4
	48	36.2±4.14	26.9±3.7
	60	30.9±3.05	29.5±2.5
		Root	
Hamidiye	12	384±19	94±10
	24	453±78	119±29
	36	568±46	173±9
	48	699±38	244±10
	60	749±44	249±19
Tokak	12	302±40	68±13
	24	353±16	92±7
	36	518±36	155±15
	48	635±70	214±24
	60	723±34	274±10

4.5. Levels of Lipid Peroxidation

The level of lipid peroxidation was measured in terms of MDA (malondialdehyde) concentration (Table 4.6). Interestingly, at nil Cd supply, Tokak had higher levels of lipid peroxidation when compared to the plants with Cd supply (Table 4.6). The reason for such higher inherent level of lipid peroxidation could not be understood. Increasing supply of Cd gradually increased lipid peroxidation in shoot of Hamidiye, but reduced in Tokak. In Hamidiye, the increases in lipid peroxidation by enhanced Cd supply were calculated to be 32 % at 15 µM Cd, 45 % at 30 µM Cd and 73 % at 60 µM Cd. This result implies that higher sensitivity of Hamidiye to Cd toxicity is associated with higher levels of peroxidation.

Table 4.6 Lipid peroxidation in shoots of 9 days-old Hamidiye and Tokak barley cultivars after 48-hour Cd application at different concentrations. Cadmium was applied before the harvest of plants. The results show the means (± SD) of three independent experiments.

Cd Supply (µM)	Lipid Peroxidation (nmol MDA g^{-1} FW)	
	Hamidiye	Tokak
0	1.93±0.26	3.64±0.36
15	2.36±0.44	3.35±0.97
30	2.80±0.59	3.00±0.47
60	3.34±0.68	2.68±0.18

4.6. Non-Protein SH-Groups

With increasing supply of Cd, concentration of non-protein SH-groups in shoots showed a clear increase (Fig. 4.4). The increases in SH groups by Cd were particularly pronounced in Hamidiye. In Hamidiye, the concentration of SH compounds increased from 77 to 272 mg g^{-1} FW with increasing Cd supply from 0 to15 µM, and reached to 315 mg g^{-1} FW at 60 µM Cd application. The increment in SH groups by 60 µM Cd was approximately 4-fold in Hamidiye (Fig. 4.4). By contrast, the increase in SH groups by Cd supply was very little in Tokak, e.g., from 130 mg g^{-1} FW at nil Cd supply to 196 mg g^{-1} FW at 60 µM Cd supply (Fig. 4.4).

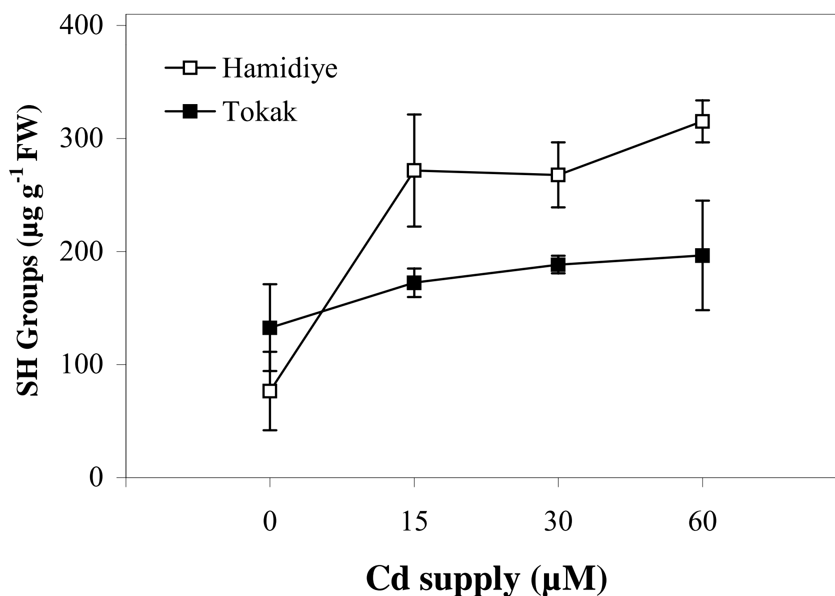

Fig. 4.4 Changes in levels of non-protein SH-compounds in leaves of 9 days-old barley cultivars subjected to increasing supply of Cd for 48 h. Cadmium was applied before the harvest of plants. The bars show the standard deviation from three independent experiments.

4.7. Ascorbic Acid Contents

Irrespective of Cd supply, Hamidiye had more ascorbic acid than Tokak (Fig. 4.5). On exposure to increasing Cd supply, ascorbic acid concentration of shoots was enhanced in Hamidiye, but did not show any consistent change in Tokak. In Hamidiye, the ascorbic acid level measured in control plants was around 2,5 mg^{-1} g FW, and increased to 3.9 mg^{-1} g FW with 60 µM Cd application. Greater amounts of ascorbic acid in Hamidiye were clearer at higher doses of Cd supply. However, it seems that the differences in ascorbic acid concentration between the both cultivars are not statistically different. The reason for irregular changes of ascorbic acid levels in Tokak in response to Cd supply could not be understood (Fig. 4.5).

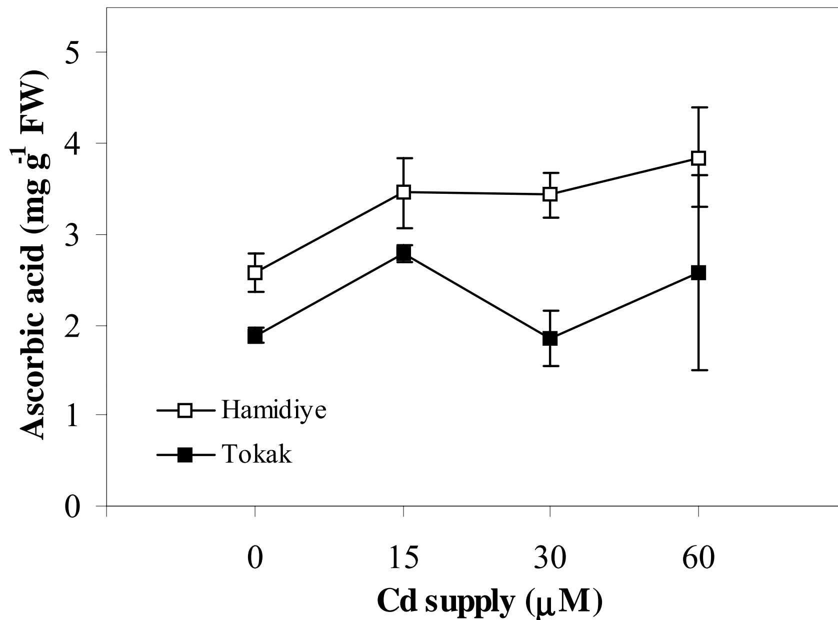

Fig. 4.5 Changes in levels of ascorbic acid in leaves of 9 days-old barley cultivars subjected to increasing supply of Cd for 48 h. Cadmium was applied before the harvest of plants. The bars show the standard deviation from three independent experiments.

4.8. Soluble Protein Concentration

Soluble protein concentrations were more or less similar between both cultivars at all doses of Cd, and not clearly affected by Cd supply (Table 4.7). In Hamidiye shoots, there was a small decrease in protein content at increasing doses of Cd. In the case of Tokak, there was a tendency for an increase in protein by Cd.

Table 4.7 Levels of soluble protein in shoots of 9 days-old Hamidiye and Tokak cultivars after increasing Cd supply for 48h. Cadmium was applied before the harvest of plants. The results show the means (± SD) of three independent experiments.

Cd Supply (µM)	Soluble Protein ($mg\ g^{-1}$ FW)	
	Hamidiye	Tokak
0	17.6±2.4	14.9±1.0
15	16.6±1.8	17.1±1.3
30	15.7±3.2	16.4±2.6
60	16.4±1.8	16.3±0.3

4.9. Ascorbate Peroxidase

Activity of ascorbate peroxidase (AP) in Tokak was not affected by increasing Cd supply, but showed an important increase in Hamidiye (Fig. 4.6).

With increasing Cd supply from 0 to 15 µM Cd supply, a 1.5-fold increase in activity (from 3.43 to 5.24 $\mu mol\ g^{-1}$ FW min^{-1}) was found in shoots of Hamidiye, while, in Tokak increase in AP by Cd was very slight. When the Cd supply was 60 µM, the AP activity was around 6 $\mu mol\ g^{-1}$ FW min^{-1} in Hamidiye showing a 73% increment. In the case of Tokak

the increase in AP activity resulted from 60 μM Cd application was only 4 % (Fig. 4.6).

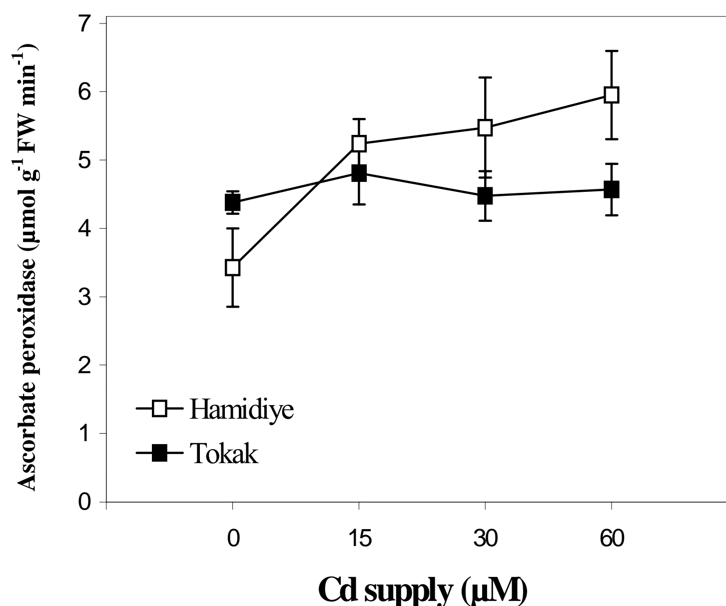

Fig. 4.6 Changes in activity of ascorbate peroxidase in shoots of 9 days-old two barley cultivars subjected to increasing Cd concentrations for 48 h. Cadmium was supplied before the harvest of plants. The bars show the standard deviation of three independent experiments.

4.10. Glutathione Reductase

The cultivars greatly differed in glutathione reductase (GR) activity when Cd was supplied. In general, Hamidiye showed a gradual enhancement in GR levels whereas Tokak exhibited a tendency to depress GR activity (Fig. 4.7). For example, increasing Cd supply from 0 to 60 μM caused an increase in GR activity by 60 % in Hamidiye, but reduced the

activity in Tokak by around 10 % (Fig. 4.7).

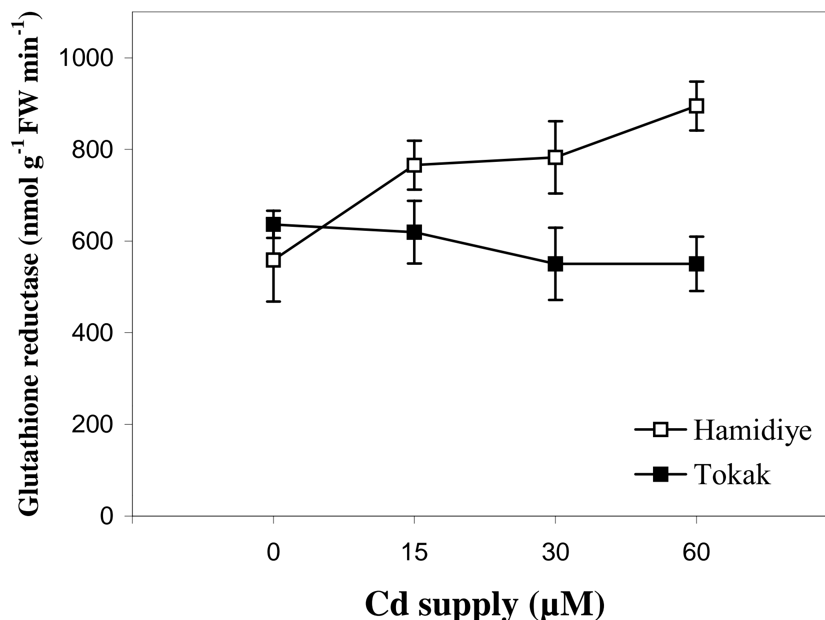

Fig. 4.7 Changes in activity of glutathione reductase in shoots of 9 days-old two barley cultivars subjected to increasing Cd concentrations for 48 h. Cadmium was supplied before the harvest of plants. The bars show the standard deviation of three independent experiments.

4.11. Superoxide Dismutase

Irrespective of Cd application superoxide dismutase (SOD) activity was always higher in shoots of Hamidiye than Tokak (Fig. 4.8). When both cultivars are subjected to

increasing Cd supply Hamidiye generally showed an increase in SOD activity, whereas in Tokak there was a tendency to reduce SOD activity (Fig. 4.8). In Hamidiye increasing Cd application from 0 to 60 μM caused 1.5-fold increase in SOD activity, while in Tokak the activity of SOD decreased by 11 % (Fig. 4.8).

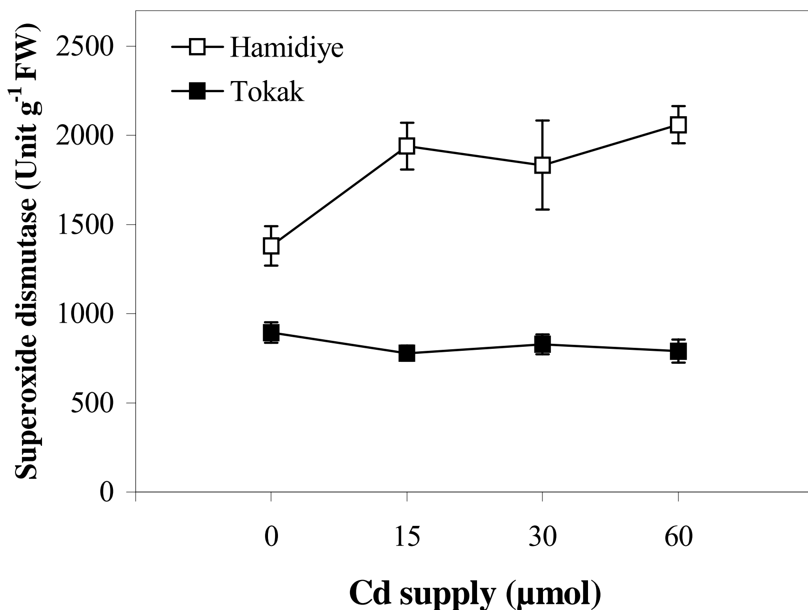

Fig. 4.8 Changes in activity of superoxide dismutase in shoots of 9 days-old two barley cultivars subjected to increasing Cd concentrations for 48 h. Cadmium was supplied before the harvest of plants. The bars show the standard deviation of three independent experiments.

4.12. Catalase

Compared to SOD, GR and AP, activity of catalase was less affected by the Cd treatments (Fig. 4.9). At the nil Cd treatment, catalase activity was higher in Tokak than Hamidiye. However, the difference in catalase activity between both cultivars became minimal with increasing Cd supply. Generally, increasing Cd supply tended to reduce and enhance activity in Tokak and Hamidiye, respectively (Fig. 4.9).

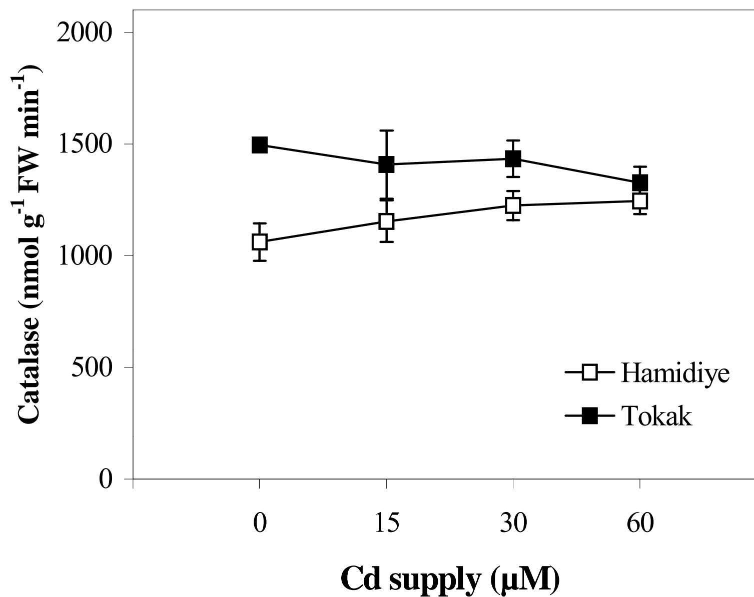

Fig. 4.9 Changes in activity of catalase in shoots of 9 days-old two barley cultivars subjected to increasing Cd concentrations for 48 h. Cadmium was supplied before the harvest of plants. The bars show the standard deviation of three independent experiments.

5. DISCUSSION

5.1. Leaf Symptoms and Growth

Exposure of plants to increasing concentrations of Cd resulted in a rapid development of chlorosis and reddish-brown discolouration of leaves and browning of roots. Occurrence of these symptoms was associated by reduction in shoot length and biomass production (Fig. 4.1). Similar phenological observations were also made by Greger and Lindberg (1987) in sugarbeet, Vazquez et al. (1989) in bean, Koleli (1998) in barley and Pandey and Sharma (2002) in cabbage under conditions of Cd toxicity. The barley cultivars Tokak and Hamidiye greatly differed in severity and development time of the symptoms. When compared to Tokak, Hamidiye was much more sensitive to increasing Cd supply, particularly regarding the severity of leaf symptoms of Cd toxicity. Before the decreases in biomass production Cd toxicity caused development of reddish-brown necrosis on older leaves of Hamidiye. Therefore, it can be suggested that appearance of leaf symptoms is the first biomarker of Cd toxicity in Hamidiye. In maize plants Lagriffoul et al. (1998) also showed that decreases in chlorophyll seems to be one of the early visible marker of Cd toxicity.

Increase in leaf chlorosis and necrosis by Cd toxicity can be a consequence of inhibition of chlorophyll synthesis and/or enhancement in chlorophyll degradation. There are several explanations for the reason of Cd-induced leaf chlorosis. Previously, Stobart et

al. (1985) showed in barley that Cd is a potent inhibitor of chlorophyll synthesis. This inhibitory action of Cd on chlorophyll synthesis is related to reduction in formation of 5-aminolaevulinic acid and protochlorophyllide reductase, which are critical in biosynthesis of chlorophyll. According to Stobart et al. (1985) Cd interacts with essential thiols (-SH groups) of protochlorophyllide reductase protein and the other proteins involved in chlorophyll biosynthesis.

Besides inhibition of chlorophyll biosynthesis, Cd was also found to be involved in oxidative degradation of chlorophyll. In studies with bean plants Somashekaraiah et al. (1992) showed that Cd causes an enhanced lipid peroxidation and consequently degradation of chlorophyll by lipid peroxides. Degradation of chlorophyll by lipid peroxides is well-known phenomena (Elstner et al., 1988; Foyer et al., 1997). As discussed below in more detail, Cd toxicity represents an oxidative stress in plants by inducing formation of highly reactive oxygen species (ROS) (Shah et al., 2001; Hegedüs et al., 2001; Vitoria et al., 2001). Particular decreases in chlorophyll by Cd were shown under light which indicates photoxidative damage of chlorophyll by ROS (Hegedüs et al., 2001). Therefore, it can be suggested that Tokak and Hamidiye differ in severity of photooxidative stress, being stronger in Hamidiye. Substantial increases in antioxidative enzymes by Cd toxicity in Hamidiye (see below for detailed discussion on this subject) support the idea that Hamidiye suffers from oxidative damage at greater level than Tokak.

Cadmium affected root growth more than shoot growth, especially at higher supply of Cd (Tables 4.1 and 4.2). A similar result was also found by Ouzounidou et al. (1997) in wheat and Vitoria et al. (2001) in radish. The reason for greater sensitivity of roots to Cd than shoots might be related to the fact that roots are the first in contact with Cd, and accumulate it at much higher amounts than shoots (Grant et al., 1998; Hegedüs et al., 2001; Vitoria et al., 2001). Also in the present study both cultivars had up to 25-fold more Cd concentration in roots than shoots (Table 4.3). Tokak and Hamidiye were not clearly different in their capacity to accumulate Cd in roots (Tables 4.3 and 4.4).

In literature, several factors have been studied as a major reason for decreases in growth by Cd toxicity, such as decreases in photosynthetic electron transport and CO_2 fixation, impaired protein synthesis and mitochondrial respiration, inhibitions in root hydraulic conductivity (inhibited water uptake) and reductions in activity of SH-containing enzymes (Greger et al., 1991; Marchiol et al., 1996; Ouzounidou et al., 1997; Krupa, 1999). Which factor mentioned is more relevant for the decreases in root and shoot growth by Cd in barley cultivars should be studied in future. But our further studies described below together with those of Hegedüs et al. (2001), Vitoria et al. (2001), Shah et al. (2001) indicate that oxidative damage catalysed by ROS seems to be an important reason for the decreases in growth by Cd toxicity.

5.2. Cadmium Uptake

Despite large differences in severity of leaf symptoms of Cd toxicity, barley cultivars had more or less similar Cd concentrations in shoots and leaves (Tables 4.3 and 4.4). Although Hamidiye tended to contain more Cd than Tokak, but this little difference in Cd accumulation cannot be a plausible reason for such distinct genotypical variation in Cd tolerance. Especially in the experiment with increasing application period of Cd, differences in shoot and root Cd concentrations between both cultivars were very similar, however leaf symptoms of Cd toxicity were more severe in Hamidiye and occurred only slightly in Tokak. These results indicate that roots or shoot concentrations of Cd are not related to differential sensitivity of barley cultivars to Cd toxicity. Our expectation was that Cd sensitive cultivar Hamidiye is sensitive to Cd because of its high capacity for root uptake and root-to-shoot translocation of Cd, because exclusion of Cd during root uptake was reported as a major plant mechanism against Cd toxicity (Grant et al., 1998; Sanita di Toppi and Gabrielli, 1999). Alternatively, retention of Cd in roots and immobilization of Cd in cell walls or compartmentalization of Cd in vacuoles of root and shoot cells might be major plant mechanisms to avoid Cd toxicity at the cellular levels. For example,

Chardonnens et al. (1998) reported that differences in Cd tolerance between ecotypes of *Silene vulgaris* were related to the preferential accumulation of Cd in vacuoles of leaf sells in Cd tolerant *S. vulgaris*. These mechanisms should be examined in future studies in Tokak and Hamidiye.

5.3. Antioxidative Defence Systems

5.3.1. Glutathione (Non-Protein SH-Groups)

When barley cultivars exposed to increasing supply of Cd, antioxidative defence systems were generally increased in Hamidiye while in Tokak there was either no consistent change or only a slight increase. As presented in Fig. 4.4, non-protein SH-compounds (thiol groups) showed marked increases in Hamidiye with the severity of Cd toxicity. The increase in level of SH-compounds in shoot of Hamidiye was around 3-fold at 60 µM Cd supply when compared to nil Cd treatment. In Tokak 60 µM Cd supply increased SH levels only by 40 %. The non-protein SH-compounds represent a major pool of glutathione. Up to 95 % of the non-protein SH-compounds comprises glutathione in different plant species (Grill et al., 1979; Maas et al., 1987). Therefore, the increases in the level of SH-compounds can be ascribed to glutathione (GSH). Glutathione exerts several important roles in protection of plants from environmental stress factors, especially in the case of Cd toxicity. Glutathione is required in the H_2O_2-scavenging ascorbate glutathione cycle which is especially located in chloroplasts (Noctor et al., 1998; Noctor and Foyer, 1999). Cadmium-induced increases in levels of SH-compounds in Hamidiye may indicate an increased activity of the ascorbate-glutathione cycle. Very recently, Mendoze-Cozatl et al. (2002) showed that treatment of *Euglena gracilis* cells with Cd resulted in up to 19-fold increases in glutathione concentration in chloroplasts. This might be considered as an

indication for Cd-enhanced H_2O_2 production in chloroplasts, and thus, activation of H_2O_2-scavenging ascorbate-glutathione cycle.

Glutathione is also required for synthesis of Cd-binding peptides such as phytochelatins (PC), which bind and sequester Cd in stable complexes in vacuoles (Cobbett, 2000; Clemens, 2001). Phytochelatins play a critical role in plant tolerance to Cd toxicity and very rapidly synthesized by exposure of plants to Cd. Importance of glutathione and PC-Cd complexes in Cd tolerance has been shown in Indian mustard (Zhu et al., 1999) and mutant lines of *Arabidopsis* (Howden et al., 1995). Similar to phytochelatins, also metallothionins (MT) are well-described metal-binding proteins involved in Cd tolerance by formation of stable MT-Cd complexes (Clemens, 2001). The role of MTs in Cd tolerance in plant cells is not well-documented, but increasing evidence suggests that transgenic plants expressing MT-genes from human or mouse cells were tolerant up to 100 µM Cd supply in growth medium (Mejare and Bülow, 2001). As the barley cultivars Tokak and Hamidiye are not clearly different in their capacity to take up and accumulate Cd in roots and shoots (Tables 4.3, 4.4 and 4.5) despite their large difference in Cd tolerance (Figs. 4.2 and 4.3), it can be suggested that Tokak and Hamidiye possibly differ in concentration of Cd-binding peptides or other compounds such as phytochelatins, metallothionins and S-containing amino acids. These compounds can contribute to better understanding the differential Cd tolerance in these cultivars. In future, studies should focus on determination of potential Cd-binding compounds in these barley cultivars by giving special attention to the expression of genes involved in synthesis of PCs and MTs. Presently, a similar study is being realized in our laboratory by K. Bilecen and U. Oztürk in the framework of their MSc studies by using two wheat cultivars differing in tolerance to Cd toxicity.

5.3.2. Ascorbic Acid

Ascorbic acid is a key antioxidant, and involved in protection of plant cells against oxidative damage catalysed by ROS. Recently, Conklin (2001) reviewed antioxidative functions of ascorbic acid. Like glutathione, ascorbic acid is essentially required in i) scavenging of H_2O_2 by ascorbate-glutathione cycle, ii) elimination of ROS, iii) used in maintenance of ∝-tocopherol (vitamin E) in reduced form, and iv) utilized as a cofactor in xanthophyll cycle to protect chloroplasts against photooxidative damage. There is little information in literature concerning the relationship between ascorbic acid and Cd toxicity or Cd tolerance of plants. In Scots pine roots Schützendübel et al. (2001) showed that total ascorbic acid concentration was initially increased in response to 12 h supply of Cd, but thereafter exhibited a rapid decline. In roots and nodules of soybean plants ascorbic acid levels were declined by increasing Cd supply (Balestrasse et al., 2001). By contrast, in the present study, in leaves of barley cultivars there was either an increase or no consistent change in total ascorbate concentration (Fig. 4.5). Upon exposure to increasing Cd supply from 0 to 60 µM Hamidiye increased ascorbate concentration by about 50 % within 48 h. This increase possibly reflects an enhanced production of ROS and thus activation of H_2O_2-scavenging ascorbate-glutathione cycle enzymes (i.e., ascorbate peroxidase and glutathione reductase). Accordingly, as discussed below in detail, these H_2O_2-scavenging enzymes showed marked increases in leaves of Hamidiye in response to Cd supply (Figs. 4.6 and 4.7). These results with ascorbic acid and SH-compounds together with the results on antioxidative enzymes (see below) support the suggestion that oxidative damage by ROS is a typical reflection of Cd toxicity in Hamidiye.

5.3.3. Antioxidative Defence Enzymes

Among the antioxidative enzymes ascorbate peroxidase (AP), glutathione reductase (GR) and superoxide dismutase (SOD) were distinctly increased in Hamidiye in response

to Cd supply, while in Tokak Cd supply remained without effect on activity of the enzymes (Figs. 4.6, 4.7 and 4.8). These increases in activity of $O_2^{\cdot -}$ - and H_2O_2- scavenging enzymes would be considered as an evidence for Cd-induced ROS production in leaves of Cd-sensitive cultivar Hamidiye. In literature there are controversial results related to the effects of Cd toxicity on AP, GR and SOD. In well agreement with the results presented in this work, Vitoria et al. (2001) found that activities of GR and SOD were increased by Cd in roots and leaves of radish plants. Increases in activity of AP by Cd have been shown in leaves of barley (Hegedüs et al., 2001) and bean (Chaoui et al., 1997). In rice plants, Cd was found to enhance $O_2^{\cdot -}$ formation and lipid peroxidation. These increases were paralleled with marked increases in SOD (Shah et al., 2001). Similarly, Dixit et al. (2001) also demonstrated increased activities of SOD, GR and AP in leaves of pea plants, and these increases were associated with enhanced levels of lipid peroxidation and H_2O_2, Therefore, Dixit et al. (2001) suggested that Cd stress results in oxidative damage by stimulating production of ROS. In view of the results reported in literature together with the results presented in Figs. 4.6, 4.7 and 4.8, it can be speculated that ascorbate-glutathione cycle is activated by Cd in Hamidiye in order to detoxify Cd-induced ROS.

Cadmium-induced increase in GR activity is also important for maintenance of glutathione in reduced form, and thus stimulation of the synthesis of Cd-binding phytochelatins. Increases in activity of GR by Cd were also reported by Chaoui et al. (1997) in bean, Ferria et al. (2002) in soybean and Fornazier et al. (2002) in sugarcane. In contrast to these results, there are studies showing decreasing or, no consistent effect of Cd toxicity on activities of AP and SOD (Gallego et al., 1999; Groppa et al., 2001; Ferreira et al., 2002; Pereira et al., 2002). In contrast to the results presented here, at least with Hamidiye, Cd toxicity was also associated with decreases in GR activity, for example in sunflower leaves (Gallego et al., 1999; Groppa et al., 2001) and poplar roots (Schützendübel et al., 2002). The reason for such inconsistent results on the effects of Cd on antioxidative defence enzymes is not known, and may be related to the differences in i) plant organ studied (root, leaf, leaf age), ii) duration and concentration of Cd used and iii) genotypes (or plant species) considered in the studies. In poplar roots, activities of

antioxidative enzymes were increased following 12 h exposure of plants to Cd, but thereafter Cd resulted rapid decrease (Schützendübel et al., 2002). Balestrasse et al. (2001) found that Cd reduced the activity of AP and GR in soybean roots when supplied at higher concentration, while at lower concentrations Cd resulted in significant increases. According to the results of Ferreira et al. (2002) exposure of soybean plants to Cd did not affect GR activity in leaves, but enhanced in roots. It seems that Cd effects are dose and plant species dependent and variable over time and plant tissue analysed. However, majority of the results indicate a general induction in activity of antioxidative enzymes with a concomitant increase in chlorophyll degradation and lipid peroxidation by increasing Cd supply. The results presented here also demonstrate that Cd effects on antioxidative defence system are also dependent on the genotype used. Activities of GR, AP and SOD were distinctly increased by Cd in Hamidiye, but not changed in Tokak. Therefore, in interpretation of the results related to the Cd effects on antioxidants special attention should be paid to the genotype used in the experiment. Depending on the genotype whether it is Cd-sensitive (like Hamidiye) or Cd-tolerant (like Tokak) the effects of Cd on antioxidants can be very variable.

In contrast to AP, GR and SOD, activity of catalase was not affected by Cd both in Hamidiye and Tokak (Fig. 4.9). This result agrees with the results of Hegedüs et al. (2001) in barley plants, but disagrees with Dixit et al. (2001), Vitoria et al. (2001) and Pereria et al. (2002) who showed marked increases in catalase activity by Cd in pea, radish and *Cratalaria juncea* seedlings, respectively. Among the enzymes studied AP, GR and SOD are predominantly localized in chloroplasts and catalase in peroxisomes (Foyer et al., 1997; Elstner et al., 1998; Foyer and Noctor, 2000). Catalase eliminates H_2O_2 produced during the photorespiration in peroxisomes. It appears that Cd toxicity did not affect H_2O_2 production during photorespiration in peroxisomes, but enhance its production during the photosynthetic electron transport.

The question is how Cd can induce production of ROS although it is a redox-inactive element in biological systems and it cannot under go a reduction/oxidation cycling to

produce ROS like redox elements Fe and Cu. There are several ways in which Cd toxicity can induce production of ROS as discussed below:

Chloroplasts are the major cell compartments producing ROS at very high rates (Asada and Takahashi, 1987; Robinson, 1988; Osmond and Grace, 1995; Foyer et al., 1997; Cakmak and Engels, 1999). During the photosynthetic electron transport a part of electrons are used for reduction of molecular O_2 leading to generation of $O_2^{.-}$ and the derivatives of $O_2^{.-}$ such as H_2O_2 and $OH^.$. Production of ROS during photosynthetic electron transport becomes pronounced when plants are exposed to environmental stress factors that diminish the utilization of absorbed light energy and photosynthetic electrons in CO_2 fixation. Under such conditions the non-utilized electrons in CO_2 fixation are transferred to O_2 leading to production of ROS (Elstner and Oswalld, 1994; Polle, 1996). By affecting photosynthetic activity of chloroplasts in different ways Cd may increase an extensive ROS production in chloroplasts. It is well-documented that Cd exerts inhibitory effects on electron flow in photosystems and CO_2 fixation, and decreases stomatal opening and activity of Calvin cycle enzymes (Weigel, 1985; Marchiol et al., 1996; Chungh and Sawhney, 1999). These changes in Cd-stressed plants can intensify use of photosynthetic electrons and absorbed light energy in activation of molecular O_2 instead of CO_2 fixation.

Alternatively, Cd can activate $O_2^{.-}$- generating NADPH-oxidase which is a membrane-bound enzyme (Bolwell and Wojtaszek, 1997; Cakmak, 2000). By interacting with membrane lipids and proteins, Cd can result in structural impairments in cell membranes with a concomitant activation of membrane-bound $O_2^{.-}$- generating NADPH oxidase. Recently, Shah et al. (2001) found an increase in formation of $O_2^{.-}$ in roots and leaves of rice plants in response to Cd treatment. Enhanced activity of NADPH oxidase by Cd was suggested as a possible reason for Cd-induced $O_2^{.-}$ formation. The role of Cd in ROS production needs to be clarified in future studies.

6. CONCLUSIONS

In the present study, two barley cultivars, Tokak and Hamidiye, were compared for their sensitivity to Cd toxicity and for the levels of antioxidants and antioxidative enzymes. The results showed existence of a wide variation in tolerance to Cd toxicity between Hamidiye and Tokak. Hamidiye is particularly affected by supply of Cd, and classified as highly Cd-sensitive cultivar, as judged from the severity of leaf symptoms and decreases in dry matter production by Cd. Although it is not as large as in Hamidiye, Cd toxicity also caused distinct decreases in dry matter production of Tokak, but could not result in a severe damage of leaves as observed in Hamidiye.

One possible reason for the differential expression of Cd tolerance between Hamidiye and Tokak might be ascribed to higher uptake and accumulation of Cd in Hamidiye. However, the results revealed that the concentration and total amount of Cd were more or less similar in both cultivars. There was a tendency for Hamidiye to take up and accumulate more Cd from growing medium, but this difference was not high enough to explain such distinct variation in sensitivity to Cd between Tokak and Hamidiye.

Higher Cd-sensitivity of Hamidiye was associated with increased levels of antioxidants and $O_2^{\cdot -}$- and H_2O_2-scavenging enzymes. By contrast, in Tokak levels of antioxidants and related antioxidative enzymes were either not affected or only slightly increased. Enhancements in activity of ROS-scavenging enzymes reflect stimulated production of ROS by Cd in Hamidiye.

Activation of ascorbate-glutathione cycle by Cd can also be beneficial for synthesis of phytochelatins because operation of this pathway at higher rate maintains glutathione in reduced form to be used in synthesis of phytochelatins. This aspect should be studied in future.

Because of very slight changes in antioxidants in response to Cd supply, in Tokak Cd is possibly detoxified by compartementation in vacuole of root and shoot cells or inactivated by formation of stable complexes with compounds such as phytochelatins or metallothionins. These points seem to be relevant for better understanding the differential Cd tolerance between barley cultivars, and therefore needed to be elucidated in further studies. In future, a special attention should be paid to the identification of the genes involved in i) tolerance to oxidative damage by ROS (i.e., genes of GR, AP and SOD) or ii) detoxification of Cd by chelation at cellular level (i.e., genes of PCs and MT

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