

Effects of Cadmium on Growth, Oxidative Stress and Antioxidant Enzyme Activities in Peanut (*Arachis hypogaea* L.) Seedlings

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Abstract

The effects of different cadmium (Cd) concentrations on growth, oxidative stress, and antioxidant enzymes activities in peanut seedlings of five cultivars were investigated in Hoagland's nutrient solution. The results indicated that the growth of seedlings and the maximal photochemical efficiency of Photosystem II were significantly reduced after treatment with 500 μM Cd^{2+} for 4 days, but no significant difference was detected when exposed to 10 and 100 μM Cd^{2+} , with the exception of FH3 being more sensitive to 100 μM Cd^{2+} . The changes in H_2O_2 and malondialdehyde contents indicated that Cd^{2+} stress caused an accumulation of reactive oxygen species and induced oxidative stress when exposed to 100 and 500 μM Cd^{2+} . The changes in total soluble protein (TSP) content and antioxidant enzyme activities were similar among the five cultivars. However, in the different concentrations of Cd^{2+} , TSP content and antioxidant enzyme activities showed different trends when compared with control groups. Exposure to 100 μM Cd^{2+} markedly enhanced activities of four antioxidant enzymes in both shoots and roots of all investigated cultivars. Additionally, cadmium stress had a more severe impact on roots as opposed to shoots of seedlings.

Keywords: *Arachis hypogaea* L., Peanut, Cadmium, Reactive oxygen species, Oxidative stress

1. Introduction

Cadmium (Cd) is an extremely significant pollutant because of its highly toxic (Sanità di Toppi and Gabbriellini 1999; Benavides et al. 2005). Its high solubility in water leads to Cd accumulation in soil and plants, and further accumulation in biological systems, which poses a major health concern for humans and animals (Järup 2002). Cd is not an essential element for plant growth, but it is readily absorbed by the roots and translocated to the upper parts of the plants (Clemens 2001; Chen et al. 2003). Although it has been shown that Cd^{2+} may have a positive effect on plant growth at low concentrations in some plants (Liu et al. 2008; Sobkowiak and Deckert 2006; Arduini et al. 2004; Aina et al. 2007), it is widely recognized as a toxic element due to its negative effects on plant growth and development. The most visible symptoms of cadmium toxicity in plants are chlorosis, stunting, browning of root tips and leaf rolls (Schützendübel et al. 2002; Chen et al. 2003; Guo et al. 2004). In general, high Cd^{2+} doses exert a serious influence on the physiological, biochemical and macromolecular properties in plants, including the inhibition of photosynthesis, a reduction in the uptake of essential mineral nutrients, an inhibition of the biosynthesis of chlorophyll, alteration of the water balance, a reduction in the activity of various enzymes and chromosomal aberrations (Clemens et al. 2002; Uraguchi et al. 2006; Ding et al. 2007; Sanità di Toppi et al. 2008).

There has been increasing evidence that suggests that the plant's response to Cd^{2+} stress has been associated with the generation of reactive oxygen species (ROS). The ROS generally stay at an acceptable level and do not cause oxidative stress due to the antioxidant defense system. However, Cd^{2+} stress can disturb the balance between the generation and removal of ROS, and cause ROS accumulation in plants which results in oxidative damage, such as lipid peroxidation (Lagriffoul et al. 1998; León et al. 2002; Lin et al. 2007; Razingar et al. 2008). To counteract the toxicity of ROS, plants have developed a defense system that functions to scavenge the cellular ROS. This system consists of enzymes including superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7) and glutathione reductase (GR, EC 1.6.4.2), and low molecular weight antioxidants such as glutathione (GSH), carotenoids, phenolics and so on (Apel and Hirt et al. 2004; Benavides et al. 2005).

In the whole plant development, the germination of seeds and the early development of seedlings are the most sensitive stages to changes in their surrounding environment (Chen et al. 2003). In this study, we focused on the early development of peanut seedlings and evaluated their tolerance to different concentrations of Cd^{2+} in hydroponics culture. The different physiological parameters, including growth of roots and shoots, photosystem II (PS II) activity, levels of oxidative stress and the antioxidant enzyme activities, were investigated in this work.

2. Materials and Methods

2.1 Plant Cultivation

Peanut (*Arachis hypogaea* L.) seeds of five cultivars including Huayu19 (HY19), Huayu22 (HY22), Huayu23 (HY23), Fenghua3 (FH3) and Luhua11 (LH11) were obtained from the Shandong Peanut Research Institute in Qingdao, China. Healthy and full seeds selected were surface sterilized with a 0.1% (v/v) sodium hypochlorite solution for 10 min and rinsed three times with distilled water. Seed capsules were peeled off with a sterile forceps. Six seeds from each cultivar were taken as a group, growing in sterile glass dishes with 50 mL Hoagland's nutrient solution containing 0, 10, 100, and 500 μM CdCl_2 . Each seed was cultured for 4 days at 20 °C with 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and using a 14:10-h light:dark photoperiod in a greenhouse. During the culture period, the shoot height and the root length were measured.

2.2 Determination of Photosystem II (PS II) Activity

The maximal photochemical efficiency of PS II (F_v/F_m) was determined using a chlorophyll fluorometer (PAM, Walz, Germany). Before the measurements, the peanut leaves were dark adapted by incubation at 20 °C for 20 min. Six seedlings from each treatment were measured. The maximal photochemical efficiency was calculated according to the following equation: $F_v/F_m = (F_m - F_o)/F_m$ (Adams and Demmig-Adams 2004). F_o was the minimum fluorescence, F_m the maximum fluorescence, and F_v the maximum fluorescence after dark adaptation and the difference between F_m and F_o .

2.3 Determination of ROS Production

To quantitatively examine the effects of the oxidative stress caused by CdCl_2 exposure, the release of ROS into the nutrient solution was determined by measuring the oxidation of 2',7'-dichlorohydrofluorescein (DCFH) to 2',7'-dichlorofluorescein (DCF) in the presence of peroxidase (EC 1.11.1.7) (Gomes et al. 2005) on a fluorometer (Bio-Rad, USA). One milliliter of media surrounding the seeds from each treatment was collected after 96 hours and added to a cuvette containing 2',7'-dichlorofluorescein-diacetate (DCFH-DA, Invitrogen, 50

μM), peroxidase (Sigma-Aldrich, 40 U mL^{-1}) and esterase (EC 3.1.1.1, Sigma, 0.45 U mL^{-1}). The cuvettes were slowly stirred for the continuous reactions, and the measurements were collected in five independent runs.

2.4 Malondialdehyde (MDA) Assay

The level of lipid peroxidation was measured by quantifying the content of MDA using thiobarbituric acid (TBA). Samples of shoots or roots (0.5 g fresh weight) were homogenized in 5 mL of 10% trichloroacetic acid (TCA). The homogenates were centrifuged at 12000 g for 10 min. After adding 2 mL of 0.6% TBA to each 2 mL aliquot of the supernatant, the mixtures were heated at 95°C for 40 min and quickly cooled in an ice bath. Then the mixture was centrifuged at 4000 g for 10 min, and the absorbance of the supernatant was recorded at 532 nm and 450 nm using the TU-1810 spectrophotometer (Pgeneral, China), respectively (Buege and Aust 1978).

2.5 Assays for Antioxidative Enzymes Activity

Samples of shoots or roots (0.1 g fresh weight) were homogenized in liquid nitrogen and immediately extracted with 1 mL of 0.05 M potassium phosphate buffer (pH 7.0) containing 0.25% (v/v) Triton X-100 and 1% (w/v) polyvinylpolypyrrolidone (PVPP) after being cultured for 4 days under CdCl_2 treatment. The extracts were centrifuged for 10 min at 12000 g at 4°C . The supernatant was used for measurement of the total soluble protein (TSP) content, SOD, CAT, POD and GR. The TSP content was obtained with Bradford's method (Bradford 1976), and the standard curve of protein concentration was described by use of standard protein solution (Jiancheng Nanjing, China). The absorbance of the solution was measured at 595 nm using TU-1810 spectrophotometer (Pgeneral, China). SOD activity was determined according to the methods described by Mishra et al. (1993). Reaction mixtures included 14.5 mM methionine, 3 μM EDTA- Na_2 , 2.25 mM nitroblue tetrazolium chloride (NBT), and 60 μM riboflavin (27:1:1:1). After adding 50 μL of enzyme extract to the 3 mL reaction mixture in tubes, the reaction was started by placing the tubes under two 15-W fluorescent lamps for 10 min and finished by placing the tubes in the dark for 10 min. The absorbance was recorded at 560 nm. One unit of SOD activity was defined as the quantity of SOD required to produce a 50% reduction of NBT in 1 mL of reaction mixture. CAT activity was measured according to the methods described by Beutler (1975). One unit of CAT activity was defined as the quantity of CAT required to decompose 1 $\mu\text{mol H}_2\text{O}_2$ per minute. POD activity was detected according to the modified method of Xu et al. (2008). One unit of POD activity was defined as the change in absorbance at 470 nm per minute. GR activity was determined according to the modified method of Ekmekci and Terzioğlu (2005). The assay mixture contained 200 mM potassium phosphate buffer (pH 7.0), 0.2 mM EDTA- Na_2 , 1.5 mM MgCl_2 , 0.5 mM GSSG, 50 μM NADPH, and enzyme extract containing 100 μg protein in a total volume of 1 mL. One unit of GR activity was defined as the quantity of GR required to cause a 1 mM decrease in the NADPH concentration per minute.

2.6 Statistics

Values cited in this paper were obtained from fully independent samples, even for nondestructive measurements (e.g., Fv/Fm measurements were not repeated on the same tissue during a time course). Differences were considered to be significant at a probability of 5% ($P < 0.05$). Tests were performed using the SPSS 13.0 statistical program.

3. Results

3.1 Seedling Growth and Fv/Fm

Shoot growth as expressed by shoot and root lengths varied depending on the Cd^{2+} concentration in the nutrient bath. Compared with the control groups, no significant difference was detected in shoot and root lengths after 4-day exposure to the 10 and 100 μM Cd^{2+} concentrations ($P > 0.05$) in HY19, HY22, HY23 and LH11, with an exception to the root growth in FH3 ($P < 0.05$, Figure 1). High concentrations of Cd^{2+} (500 μM) significantly retarded the growth of the seedlings in all five of the cultivars ($P < 0.05$) and resulted in the browning and malformation of the root tips in all five peanut cultivars. The browning of roots was not observed at 10 and 100 μM Cd^{2+} in the 4-day test.

At 10 μM Cd^{2+} , Fv/Fm did not differ significantly from the control groups in all five of the cultivars ($P > 0.05$, Figure 2). When exposed to 100 μM Cd^{2+} , the significant decline in Fv/Fm was only detected in FH3 ($P < 0.05$). However, a high concentration of Cd^{2+} (500 μM) significantly decreased the value of Fv/Fm in all five of the cultivars ($P < 0.05$).

3.2 ROS Production and Lipid Peroxidation

The exposure of peanut seedlings to Cd^{2+} resulted in the oxidation of DCFH to DCF in comparison to the control group, which indicates the production of ROS under Cd^{2+} stress in all five of the cultivars (Figure 3). After a

96-h exposure to 10 μM Cd^{2+} , H_2O_2 content did not markedly increase in all of the cultivars ($P > 0.05$). When seedlings were exposed to 100 and 500 μM Cd^{2+} , the increase in H_2O_2 content correlated to the Cd^{2+} concentration ($P < 0.05$), indicating that the higher concentrations of Cd^{2+} increased the production of ROS.

Malondialdehyde (MDA) content was measured to evaluate the level of lipid peroxidation. Ten μM of Cd^{2+} resulted in a slightly increased content of MDA in the crude extracts of both shoots and roots in comparison with the control groups, but there were no statistically significant differences ($P > 0.05$, Figure 4). Exposure to 100 and 500 μM Cd^{2+} increased the MDA content in both shoots and roots of all five of the peanut cultivars ($P < 0.05$).

3.3 Activities of Antioxidant Enzymes

In shoots, 10 μM Cd^{2+} significantly increased the activity of POD ($P < 0.05$) in all five cultivars, but the activity of SOD, CAT and GR remained unchanged when compared with the related control groups ($P > 0.05$, Figure 5, A1-A4). The exposure to 100 μM Cd^{2+} markedly enhanced the activities of four enzymes studied. At 500 μM Cd^{2+} , the activity of SOD, CAT and GR were significantly increased ($P < 0.05$), and the activity of POD was unchanged ($P > 0.05$) in all five cultivars when compared to the control groups.

There were no significant differences detected in SOD and GR activity after the 4-day treatment of 10 μM Cd^{2+} in the roots of all five cultivars ($P > 0.05$, Figure 5, B1-B4). But this Cd^{2+} level caused a significant increase in the activity of CAT and POD ($P < 0.05$). In the presence of 100 μM Cd^{2+} , the activity of the antioxidant enzymes studied were increased in the roots ($P < 0.05$). However, the treatment with 500 μM Cd^{2+} did not change the activity of SOD and CAT ($P > 0.05$) but did decrease the activity of POD and GR ($P < 0.05$).

4. Discussion

4.1 Disturbances in the Growth and Photosynthetic Rate

High Cd^{2+} concentrations (500 μM) resulted in a reduction in seedlings growth, expressed as shoot and root length, induced root browning and inhibited PS II activity in all five of the cultivars. No visual symptoms from Cd^{2+} toxicity were detected at 10 and 100 μM Cd^{2+} . However, cultivar FH3 was sensitive to 100 μM Cd^{2+} for root growth and in Fv/Fm values. The inhibition of growth induced by Cd^{2+} was mainly due to the effect on photosynthesis rate (Sandalio et al. 2001). The inhibition of biomass production and the rate of photosynthesis from Cd^{2+} stress has been previously reported in other plants species (Lin et al. 2007; Hayat et al. 2007; Xu et al. 2008). Shi and Cai (2008) reported that Cd^{2+} treatment (10, 50, 100 μM) caused an inhibition in the net photosynthetic rate of 14-d-old peanut seedlings. In our study, there was no significant difference in Fv/Fm values found at 10 and 100 μM Cd^{2+} in all five cultivars, which may be attributed to the relationship between the growth stage and the sensitivity toward Cd^{2+} stress.

It has been previously reported that low levels of Cd^{2+} can have a positive effect on plant growth (Sanità di Toppi and Gabbrielli 1999; Lin et al. 2007). However, the stimulatory effect of Cd^{2+} on the growth of the peanut seedlings was not observed in the dose range tested in this study. In addition, the inhibition of seed germination was not observed in all five of the peanut cultivars, even in the presence of high concentrations of Cd^{2+} (500 μM). This result was similar to the results observed in wheat, barley and rice (Titov et al. 1995; Rascio et al. 2008).

4.2 ROS Accumulation and Lipid Peroxidation

The increase in the fluorescence of DCF indicated that there was a production of ROS resulting from Cd^{2+} stress. H_2O_2 , a form of ROS, is uncharged and readily diffuses across biological membranes into the nutrient solution (Lesser 2006) and reacts with DCFH to produce DCF, which can be easily detected (Gomes et al. 2005). A comparison of H_2O_2 content among the five cultivars showed that 100 and 500 μM Cd^{2+} resulted in a sustained increase in fluorescence, indicating the accumulation of ROS induced by Cd stress. The accumulation of ROS further increased the level of lipid peroxidation in both shoots and roots of peanut cultivars.

It is known that the balance of the steady-state levels of ROS is determined by the interplay between different ROS-producing and ROS-scavenging agents (Seregin and Ivanov 2001; Benavides et al. 2005). Some studies showed that the homeostasis of ROS is interrupted by Cd^{2+} stress in plants (Sanità di Toppi and Gabbrielli 1999; Benavides et al. 2005). The continued presence of ROS would have detrimental effects on the peanut seedlings if they could not be rapidly removed (Lesser 2006; Clemens 2006). In this study, 100 and 500 μM Cd^{2+} increased the production of ROS; the latter was parallel to the degree of lipid peroxidation and related to the changes in growth and photosynthesis rates. Similar trends have been reported in other species (Lagriffoul et al. 1998; Sandalio et al. 2001; Schützendübel et al. 2002; Lin et al. 2007).

4.3 Changes in ROS-related Enzymes

To defend against oxidative stress and scavenge ROS, plants possess a well-organized antioxidant defense system that contains antioxidative enzymes and antioxidants (Apel and Hirt 2004; Hall 2002). The cooperative function of these antioxidative enzymes and low molecular weight antioxidants play an important role in the scavenging of ROS and maintaining the physiological redox state of the organism (Lesser 2006).

At 10 μM Cd^{2+} , activity of POD in shoots and activity of CAT and POD in roots increased to cope with Cd^{2+} stress, but this concentration of Cd^{2+} did not result in an accumulation of ROS. It is likely that oxidative stress was inhibited by the function of defensive system in this stage of the peanut seedling. However, 100 μM Cd^{2+} caused oxidative stress and increased the activity of enzymes studied in both shoots and roots. The increased activity of SOD, CAT, POD and GR caused by Cd^{2+} has been previously observed in several plant species and is considered to be an adjustment response to stress (Lagriffoul et al. 1998; Dinakar et al. 2008; Xu et al. 2008).

At the highest concentration of Cd^{2+} (500 μM) tested, the activity of all four antioxidative enzymes tested were inhibited in roots, whereas in shoots, only POD was inhibited. As previously reported in other plants, high concentrations of Cd^{2+} resulted in serious phytotoxicity (Lagriffoul et al. 1998). Cd^{2+} accumulation in plants and Cd-induced inhibition of orderly physiological functions were also associated with H_2O_2 accumulation and growth retardation in plant development (Benavides et al. 2005). The exposure to high Cd^{2+} concentrations might directly cause the inhibition of the antioxidative enzymes by Cd^{2+} binding (Chen et al. 2003; Clemens 2006; Hayat et al. 2007).

Cadmium stress induced more damage to roots than to shoots in the five peanut cultivars studied. This conclusion is based on the concentrations of H_2O_2 and MDA, on the varying patterns of the activity of the antioxidative enzymes in different Cd^{2+} concentrations. Roots were directly exposed to Cd^{2+} in the medium, while shoots were not directly exposed and might be less impaired by oxidative stress.

The differences in responses to Cd are surprisingly small among the five cultivars. The result might indicate that the five cultivars in this study were all Cd-sensitive genotypes (Metwally et al. 2005). However, we found FH3 was more sensitive to Cd^{2+} stress than the other four cultivars. Further investigation is needed to illuminate whether this sensitivity is due to the genotypic and ecotypic differences of the peanut cultivars.

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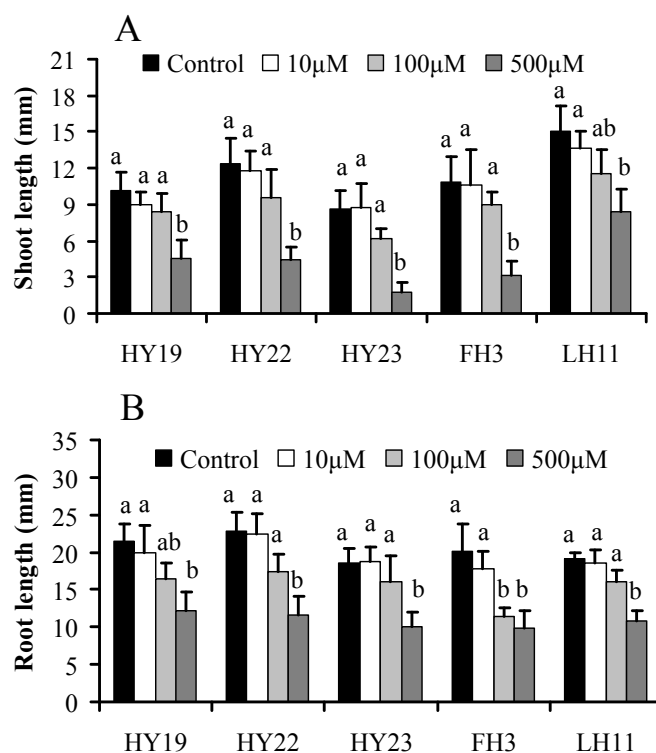


Figure 1. Shoot (A) and root (B) lengths of the five peanut (*Arachis hypogaea* L.) cultivars exposed to different Cd concentrations. Vertical bars are the standard deviations (n = 6). Values were compared within different Cd concentrations in the same cultivar. Those that were significantly different ($P < 0.05$) are indicated by different letters

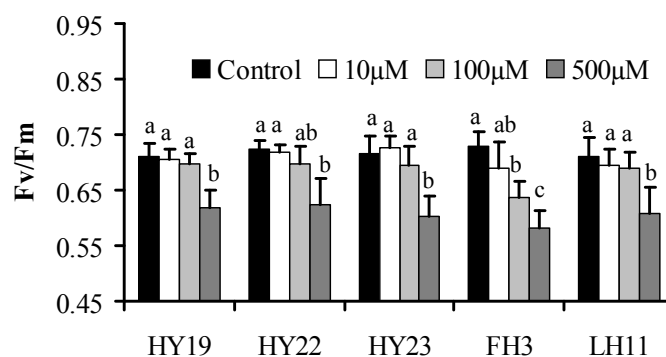


Figure 2. The maximal photochemical efficiency of PS II (Fv/Fm) of young seedlings from five peanut cultivars exposed to different Cd concentrations. Vertical bars are the standard deviations (n = 6). Values were compared within different Cd concentrations in the same cultivar and significant differences ($P < 0.05$) are indicated by different letters

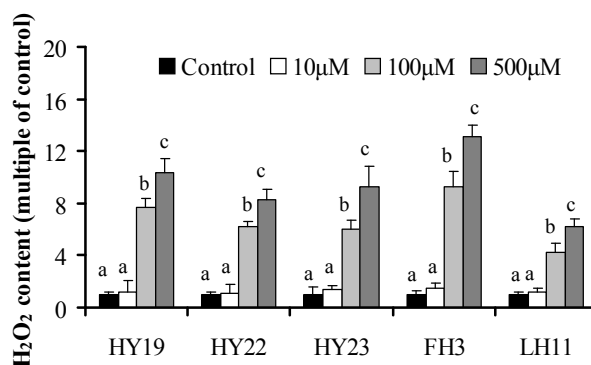


Figure 3. ROS content (A) measured as the increase in DCF fluorescence upon exposure to different Cd concentrations. ROS content was expressed as a multiple of the control. Vertical bars are standard deviations (n = 5). Values were compared within different Cd concentrations in the same cultivar and significant differences ($P < 0.05$) are indicated by different letters

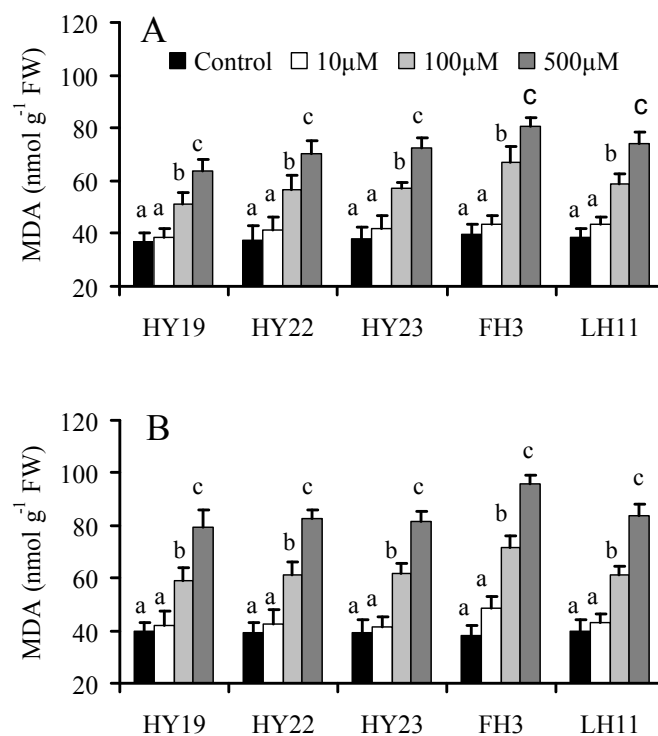


Figure 4. MDA content in shoots (A) and roots (B) of five different peanut cultivars after 4-day exposure to Cd. Vertical bars are standard deviations ($n = 4$). Values were compared within different Cd concentrations in the same cultivar and significant differences ($P < 0.05$) are indicated by different letters

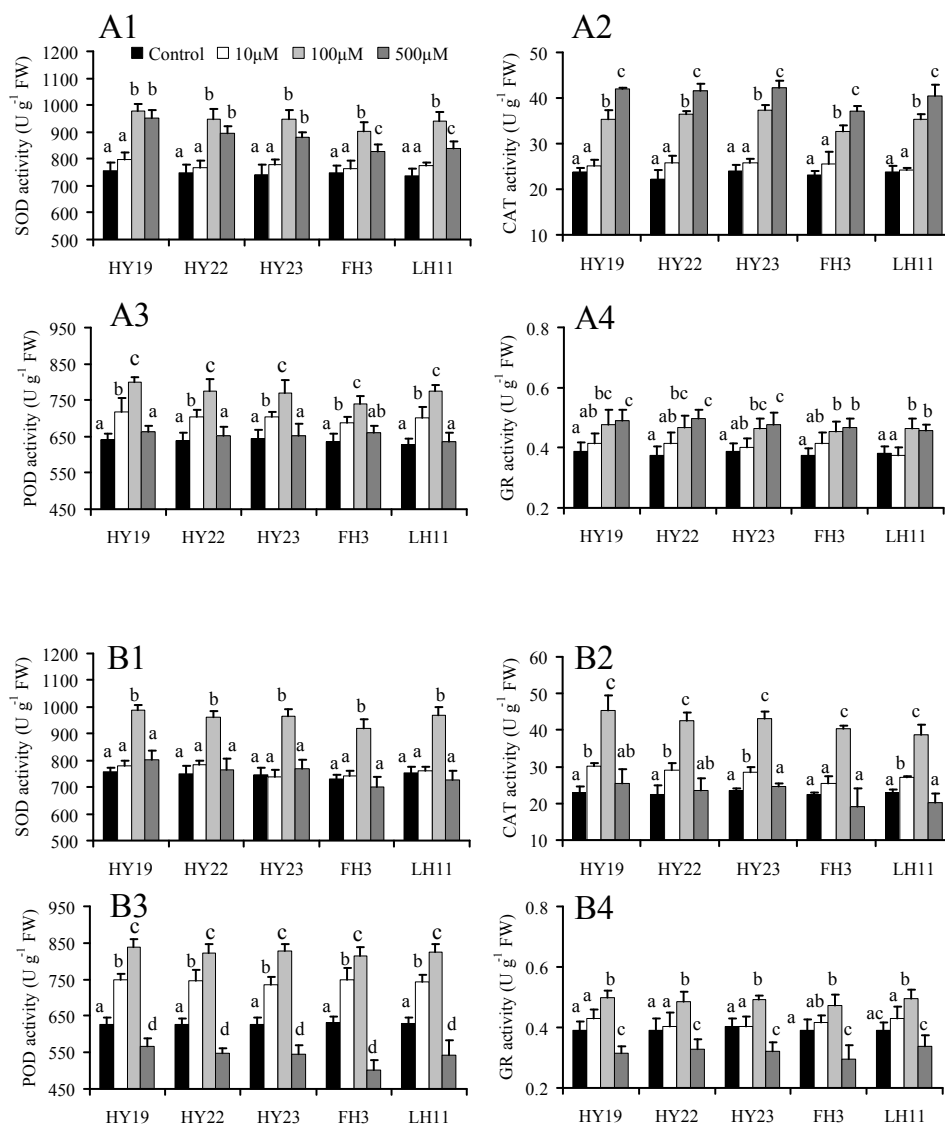


Figure 5. The activity of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and glutathione reductase (GR) in the shoots (A1-A4) and the roots (B1-B4) exposed to different Cd concentrations. Vertical bars are standard deviations (n = 4). Values were compared within different Cd concentrations in the same cultivar and significant differences ($P < 0.05$) are indicated by different letters