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BRIEF COMMUNICATION

# Interactive effects of cadmium and aluminum on growth and antioxidative enzymes in soybean

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## Abstract

The effects of Al, Cd and pH on growth, photosynthesis, malondialdehyde (MDA) content, and some antioxidant enzyme activities of the two soybean cultivars with different Al tolerance were determined using a hydroponic culture. There were six treatments as follows: pH 6.5; pH 4.0; pH 6.5 + 1.0 μM Cd; pH 4.0 + 1.0 μM Cd; pH 4.0 + 150 μM Al; pH 4.0 + 1.0 μM Cd + 150 μM Al. The results showed that the low pH (4.0) and Al treatments caused marked reduction in the growth (root and shoot length and dry mass), chlorophyll content (SPAD value) and net photosynthetic rate. Higher malondialdehyde content, superoxide dismutase (SOD) and peroxidase (POD) activities were detected in the plants exposed to both Al and Cd than in those exposed to Al treatment alone. An expressive enhancement of SOD and POD was observed in the plants exposed to 150 μM Al in the comparison with the control plants, especially in Al-sensitive cv. Zhechun 2 which had also significantly higher Al and Cd content than Al tolerant cv. Liao-1. Cd addition increased Al content in the plants exposed to Al + Cd stress, and cv. Zhechun 2 had relatively lower Al content. The present research indicated that Al and Cd are synergistic in their effects on plant growth and some physiological traits.

*Additional key words*: *Glycine max* L., peroxidase, photosynthesis, superoxide dismutase, tolerance.

Cadmium (Cd) is a toxic heavy metal which causes phytotoxicity, and its uptake and accumulation in plants moves it further into food chain, leading to a potential threat to human health (Shah and Dubey 1998). Although not essential for plant growth, Cd ions are readily taken up by roots and translocated to above-ground parts. While Cd toxicity for plants has been proven to be a major environmental problem, the mechanism of its action has not been fully investigated. The presence of excessive amount of Cd in soil may cause many physiological disorders in plants such as inhibition of seed germination, reduction of growth especially the root growth, disturbances in mineral nutrition and sugar metabolism and therefore, strongly influences biomass production (Sanita di Toppi and Gabbrielli 1999). Cd toxicity is correlated with alterations in the functionality of membranes due to changes in lipid composition and reduction of enzymatic activities associated with membranes (Fodor *et al*. 1995), decrease in photosynthetic rate due to reduced chlorophyll content and the enzymatic activity involved in $CO_2$ fixation (Greger and Ögren 1991). In many plants Cd enhances the level of lipid peroxidation and alteration in antioxidant systems (Somashekaraiah *et al*. 1992).

Aluminum is one of the most abundant elements in the earth crust only after oxygen and silicon, comprising about 7 % of its mass (Kochian 1995). The primary Al toxicity symptom observed in plants is inhibition of root growth (Delhaize and Ryan 1995 Kochian 1995), followed by less nutrient and water absorption, resulting in poor growth and production. Al interferes with uptake, transport and utilization of essential nutrients including Ca, Mg, K, P, Cu, Fe, Mn and Zn (Foy 1984, Guo *et al*. 2003). The important limiting factor for the unfavorable growth of many crops in acid soils is the Al-toxicity induced by the large amounts of soluble aluminum, which causes damage of structure and function of cell membrane (Kappus 1987).

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*Abbreviations*: Chl - chlorophyll; $g_s$ - stomatal conductance; MDA - malondialdehyde; $P_N$ - net photosynthetic rate; POD - peroxidase; SOD - superoxide dismutase.
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Interaction between Cd and other nutrients would lead to the changes in nutrient content of the plants and physiological disorders as well as retardation of growth and yield (Agrawal and Sharma 2006, Österås and Greger 2006). It has been demonstrated that Cd availability increases with decreased soil pH (Wu and Zhang 2002). However, little has been known on combined effect of Cd and Al on plants, although their individual toxicity has already been established.

The objectives of this study were to investigate *1*) the effect of low pH and Al toxicity on growth and antioxidative enzyme activities in different soybean cultivars, *2*) the effect of toxic Cd concentration on the response of soybean to Al, and *3*) the combined effects of Cd and Al on soybean cultivars.

Two soybean (*Glycine max* L.) cultivars, Z2 (Z2; Al-sensitive) and L1 (L1; Al-tolerant) were grown in hydroponic solution under the controlled conditions (12-h photoperiod, irradiance of 220 μmol $m^{-2}$ $s^{-1}$, and day/night temperature of 28/20 °C). The composition of the basic nutrient solution was (mg $dm^{-3}$): $NH_4NO_3$ - 58.03, $NaH_2PO_4 . 2\ H_2O$ - 24.99, $K_2SO_4$ - 87.13, $MgSO_4$ - 61.05, $CaCl_2$ - 55.49, Fe-citrate - 4.47, $MnCl_2 . 4\ H_2O$ - 0.45, $ZnSO_4 . 7\ H_2O$ - 0.22, $CuSO_4 . 5\ H_2O$ - 0.04, $HBO_3$ - 2.9, $H_2MoO_4$ - 0.01. One week after transplanting to the basic culture solution, Cd as $CdCl_2$ and Al as $AlCl_3 . 6\ H_2O$ were added to the corresponding containers, and the solution pH was adjusted with HCl to form the following six treatments each with three replications: T1 - pH 6.5; T2 - pH 4.0; T3 - pH 6.5 + 1.0 μM Cd; T4 - pH 4.0 + 1.0 μM Cd; T5 - pH 4.0 + 150 μM Al; T6 - pH 4.0 + 1.0 μM Cd + 150 μM Al. The experiment was laid out according to completely randomized design (CRD). The pH of solution in each container was adjusted every other day with HCl or NaOH as required. The nutrient solution in the growth containers was continuously aerated with pumps and renewed every 5 d.

At the 20$^{th}$ day after treatment, the second fully expanded leaves were selected for measuring chlorophyll (Chl) content with a chlorophyll meter (*Minolta* SPAD-502, Osaka, Japan) and photosynthetic parameters with an infra red gas analyzer (*LI-6400*, *Li-COR*, Lincoln, USA). The net photosynthetic rate ($P_N$) was measured at $CO_2$ concentration of 340 - 360 μmol $mol^{-1}$, relative humidity 50 - 60 %, temperature 28 - 32 °C and photosynthetically active radiation (PAR) of 1500 μmol $m^{-2}$ $s^{-1}$.

At the 28$^{th}$ day after treatment, the upper second fully expanded leaves were sampled for the analysis of relevant enzymes. The samples were washed with distilled water and ground with a pestle and mortar under chilled conditions in a buffer specific for each enzyme. The homogenate was filtered through four layers of muslin cloth, and centrifuged at 3 000 *g* for 20 min at 4 °C, and the supernatants were used for enzyme assays.

The superoxide dismutase (SOD; E.C.1.15.1.1) activity was assayed according to Beauchamp and Fridovich (1971) with some modification. The assay mixture in contained 50 mM phosphate buffer, pH 7.8, 9.9 mM L-methionine, 57 μM nitroblue tetrazolium (NBT), 0.025 % (m/v) *Triton X-100*, and 0.0044 % (m/v) riboflavin. The photoreduction of NBT (formation of purple formazan) was measured at 560 nm using spectrophotometer (UV-2450, Shimadzu, Japan) and one unit of SOD was defined as that being present in the volume of extracts that caused inhibition of photoreduction of NBT by 50 %.

Peroxidase (POD; EC 1.11.1.7) activity was measured by the method of Chandlee and Scadalios (1984). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.1), 1 % guaiacol, 0.4 % $H_2O_2$ and enzyme extract. Increase in the absorbance due to oxidation of guaiacol (coefficient of absorbance 25.5 $mM^{-1}$ $cm^{-1}$) was measured at 470 nm. Enzyme activity was calculated in terms of μmol(guaiacol oxidized) $g^{-1}$(f.m.) $min^{-1}$ at 25 ± 2 °C.

The level of lipid peroxidation was expressed as malondialdehyde (MDA) content and was determined as 2-thiobarbituric acid (TBA) reactive compounds. Plant fresh tissues (0.2 g) were homogenized and extracted in 10 $cm^3$ of 0.25 % (m/v) TBA made in 10 % (v/v) trichloroacetic acid. Extract was heated at 95 °C for 30 min and then quickly cooled on ice. After centrifugation at 10 000 *g* for 10 min, the absorbance of the supernatant was measured at 532 nm using coefficient of absorbance 155 mM $cm^{-1}$.

Al and Cd concentration in both roots and shoots were determined by an atomic absorption spectroscopy (Shimadzu, Japan) after the samples were dry ashed in a muffle furnace and prepared with a solution of $HNO_3$ and $H_2O$ (1:1).

The measurements were done with three replicates and statistical analyses were carried out by one-way *ANOVA* using Student's *t*-test to compare the significance of difference between the treatments (Steel and Torrie 1980).

The effect of Al and Cd stresses on plant growth was evaluated in terms of shoot and root length and dry mass (Table 1). There was an obvious difference in these growth parameters between the two genotypes, with L1 having larger values than Z2 in all the treatments. In comparison with control variant (pH 6.5), the treatment pH 6.5 + 1.0 μM Cd resulted in a slightly fast growth, indicating the stimulating effect of moderate Cd content on soybean growth. At pH 4.0, the growth inhibition was more severe in the treatment of 150 μM Al addition than in the treatment of 1.0 μM Cd addition. Meanwhile there was a significant difference in growth inhibition between pH 6.5 and pH 4.0 treatments with the same Cd level, indicating the association of Cd toxicity and acidity in the medium. More pronounced and statistically significant reduction in all growth parameters of both the genotypes was observed in the treatment of combined application of Cd and Al at pH 4.0. This indicated that Al and Cd are synergistic in toxic effect on plant growth.

Two soybean cultivars grown in control conditions had no distinct difference in Chl content, $P_N$ and stomatal

conductance ($g_s$) (Table 2). Addition of Cd into the solution of pH 6.5 did not cause significant decline in these parameters. In comparison with the plants exposed to the solution of pH 6.5, the plants grown in the solution of pH 4.0 had smaller values and addition of Cd resulted in reduced Chl content, $P_N$ and $g_s$ especially in Z2. With the same pH 4.0, addition of 100 μM Al caused greater decline in the examined three parameters than addition of 1.0 μM Cd. The greatest reduction in these parameters could be found in the combined treatment of Al and Cd.

The contents of Al and Cd were higher in the soybean roots as compared with those in shoots (Table 1), however, the changes due to pH and heavy metal application were similar. In comparison with pH 6.5, lower pH (4.0) resulted in a significant increase in root and shoot Al content in both genotypes. Addition of Al increased the Al concentration in both plant parts dramatically. Without Al addition, Cd treatment tended to reduce Al content in plant parts, whereas in the presence of Al, Cd treatment increased Al content in roots but decreased Al content in of shoots. Cd contents in roots and shoots were significantly higher under pH 4.0 as compared with pH 6.5. Addition of Al alone at pH 4.0 caused a slight decrease in Cd content; however, there was a not significant increase when Cd and Al were added together. The Al-tolerant cultivar L1 had much lower Al and Cd content in roots and shoots than relatively sensitive cultivar Z2.

The final product of membrane lipids peroxidation is malondialdehyde (MDA), which accumulates when plants are subjected to oxidative stress. Therefore, the concentration of MDA is commonly considered as a general indicator of lipid peroxidation (Chaoui *et al*. 1997). All stress treatments increased MDA content and the combined addition of Cd and Al to the solution with pH 4.0 resulted in the greatest increase, followed by Al addition alone. The increase of MDA content by stress treatments was greater in Z2 than in L1.

Table 1. Effect of the different stress treatments on growth characteristics and contents of Al and Cd in two soybean cultivars. Different letters after data within a column represent significant difference at 95 % probability.

| Cultivar | Treatment | Length [cm] | | Dry mass [g plant$^{-1}$] | | Al [μg g$^{-1}$(d.m.)] | | Cd [μg g$^{-1}$(d.m.)] | |
|---|---|---|---|---|---|---|---|---|---|
| | | shoot | root | shoot | root | shoot | root | shoot | root |
| L1 | pH 6.5 | 29.5ab | 32.2a | 3.59a | 2.20ab | 61.2c | 29.0c | 10.6d | 3.4d |
| | pH 4.0 | 25.9abc | 30.6a | 3.39a | 2.15abc | 108.1b | 51.2b | 24.8c | 6.2c |
| | pH 6.5 + $Cd^{2+}$ | 34.7a | 37.5a | 3.70a | 2.25a | 52.1c | 25.9c | 53.5b | 7.8b |
| | pH 4.0 + $Cd^{2+}$ | 21.1bc | 28.5a | 3.27a | 2.15abc | 95.3b | 45.3b | 66.2a | 9.6a |
| | pH 4.0 + $Al^{3+}$ | 20.9bc | 27.9a | 2.70b | 2.05bc | 783.9a | 209.3a | 21.4c | 5.7c |
| | pH 4.0 + $Cd^{2+}$ + $Al^{3+}$ | 19.3c | 18.3b | 2.49b | 2.00c | 811.5a | 197.8a | 71.9a | 10.7a |
| Z2 | pH 6.5 | 22.8b | 23.2b | 2.91b | 2.15ab | 76.8c | 32.4c | 12.7d | 4.5d |
| | pH 4.0 | 20.1b | 22.7b | 2.74b | 2.05bc | 124.3b | 68.2b | 27.2c | 7.8c |
| | pH 6.5 + $Cd^{2+}$ | 29.2a | 33.9a | 3.40a | 2.30a | 65.9c | 27.2c | 62.1b | 9.4b |
| | pH 4.0 + $Cd^{2+}$ | 17.6bc | 16.4c | 2.65b | 2.00bc | 109.8b | 56.1b | 74.0a | 11.2a |
| | pH 4.0 + $Al^{3+}$ | 16.7bc | 16.0c | 2.17bc | 2.00bc | 897.8a | 241.9a | 23.7c | 7.3c |
| | pH 4.0 + $Cd^{2+}$ + $Al^{3+}$ | 12.0c | 15.0c | 1.99c | 1.90c | 926.9a | 217.1a | 79.8a | 12.6a |

Table 2. Effect of the different stress treatments on chlorophyll content, net photosynthetic rate, stomatal conductance, MDA content and activity of two antioxidant enzymes in two soybean cultivars. Different letters after data within a column represent significant difference at 95 % probability.

| Cultivar | Treatment | Chl content (SPAD) | $P_N$ [μmol m$^{-2}$ s$^{-1}$] | $g_s$ [mol m$^{-2}$ s$^{-1}$] | MDA content [μmol g$^{-1}$(f.m.)] | SOD [U g$^{-1}$ (f.m.)] | POD [μmol g$^{-1}$(f.m.) min$^{-1}$] |
|---|---|---|---|---|---|---|---|
| L1 | pH 6.5 | 33.7a | 11.6a | 0.37a | 12.7c | 156.4c | 25.1c |
| | pH 4.0 | 31.8ab | 10.0a | 0.27ab | 18.6bc | 176.5b | 33.5b |
| | pH 6.5 + $Cd^{2+}$ | 33.0a | 11.1a | 0.35ab | 15.0c | 161.5c | 28.1c |
| | pH 4.0 + $Cd^{2+}$ | 30.2ab | 10.8a | 0.25ab | 21.9b | 185.2b | 36.9b |
| | pH 4.0 + $Al^{3+}$ | 29.6ab | 4.8b | 0.19ab | 25.4ab | 190.4ab | 38.2ab |
| | pH 4.0 + $Cd^{2+}$ + $Al^{3+}$ | 26.7b | 4.7b | 0.16b | 28.6a | 203.5a | 43.9a |
| Z2 | pH 6.5 | 35.6a | 11.3a | 0.34a | 15.7d | 185.3d | 28.4d |
| | pH 4.0 | 31.4ab | 8.7bc | 0.29ab | 21.4cd | 205.9c | 34.9c |
| | pH 6.5 + $Cd^{2+}$ | 32.4ab | 10.9ab | 0.31ab | 19.0d | 194.7cd | 31.0cd |
| | pH 4.0 + $Cd^{2+}$ | 28.3bc | 7.3c | 0.26ab | 24.7c | 220.6bc | 37.5bc |
| | pH 4.0 + $Al^{3+}$ | 25.5c | 7.1c | 0.23b | 30.2b | 232.2b | 41.7b |
| | pH 4.0 + $Cd^{2+}$ + $Al^{3+}$ | 16.9d | 5.1d | 0.19ab | 36.3a | 258.2a | 47.9a |

SOD is an essential component of antioxidative defense system, as it dismutates two $O_2^-$ to $H_2O_2$ and oxygen. Plants exposed to low pH, Al alone and combined Al with Cd showed a significant increase in the activity of SOD relative to pH 6.5 control (Table 2). Peroxidase (POD) is also an important enzyme against oxidative stress, being able to scavenge $H_2O_2$, which is a major product produced by SOD. The POD activity was also increased and the combined addition of Cd and Al caused a substantially higher POD activity. Furthermore, there was a significant genotypic difference in the enhancement of POD activity. L1 had significantly lower POD values compared to Z2.

The growth of soybean genotypes in terms of root/shoot length and dry mass was not affected significantly by 1.0 μM Cd, and a little increase was observed at pH 6.5. It was due to the fact that Cd level in the solution culture was quite low. The result is in agreement with the previous findings that there is some potentially positive effect of Cd on plant growth at lower metal concentration (Wu and Zhang 2002, Guo *et al*. 2004). The growth reduction by Al (150 μM) was more pronounced as compared with Cd (1 μM). It might be due to the reason that at low pH the toxicity of Al to plants is enhanced. The growth inhibition by Al stress may be related to several biochemical processes. In the plant species of Al non-accumulators, the negative effect of Al on plant growth prevails in soils with low pH (Marschner 1995), the reduction of root growth being the most serious consequence (Tabuchi and Matsumoto 2001). Lidon and Barreiro (2002) observed that Al toxicity decreased significantly the concentrations of N, Mg, P and Fe, and hypothesized that P deficiency specifically triggers the reduction of biomass production. However there has been little understanding of combined effects of Cd and Al on plants. In the current study, the growth inhibition was more severe in Cd +Al than in Al alone treatment, indicating that the effect of Cd and Al is synergistic. Guo *et al*. (2004) also demonstrated that seedling growth in terms of dry mass was dramatically inhibited with low pH and Al treatments alone and combined with Cd. However, when Cd was added at the same concentration to the solution with Al at pH 4.0, the inhibition of plant growth was further enhanced compared to the treatment without Cd. Moreover, Al-sensitive cultivar Z2 was more inhibited than Al-tolerant cultivar L1.

Chl content, $P_N$ and $g_s$ were affected negatively by Cd and Al, especially when both were applied in the same treatment. It is long recognized that Al toxicity specifically inhibits the photosynthetic apparatus of many species (Akaya and Takenaka 2001). Ashraf and Bashir (2003) found that salt stress also caused a marked reduction in $P_N$, transpiration rate and $g_s$ in the species of *P. vulgaris* than in *S. aculeata*.

In the present study, addition of Al alone or in combination with Cd increased MDA content and activity of SOD and POD significantly, while the effect of low pH and Cd treatments was not significant. SOD in leaves, roots and stolons were increased in the presence of $Cd^{2+}$ when compared to control plants of *Phragmites* (Iannelli *et al*. 2002). POD induction is a general response of vascular plants to uptake of toxic amounts of metals. It has been observed in roots and leaves of various species after application of toxic doses of Zn, Cd, Cu, Ni and Pb (Van Assche and Clijsters 1990). Previously, Ezaki *et al*. (1996) reported that a anionic POD was activated by Al stress in tobacco cells. It appears that toxic metals change POD activity both quantitatively and qualitatively. It also appears that the increase in POD activity is a defensive response to most if not all metals, which may cause damage or disturb normal function of the plants. The results are in agreement with the findings reported by Hassan *et al*. (2005).

The low pH, Al and Cd treatments affected Al and Cd concentrations of plant roots/shoots significantly. Al concentration in plants under pH 4.0 + $Cd^{2+}$ + $Al^{3+}$ treatment was much higher than that of Al treatment alone, showing the stimulation of Al uptake by Cd. Similarly, higher Cd concentration were recorded when Cd was applied with Al, showing the synergistic relationship of both elements in Cd and Al uptake. Moreover, L1 had lower Al and Cd contents than Z2. The results are in agreement with Kidd and Proctor (2000) who reported that Al concentration of Al-sensitive *Betula pendula* was higher in roots but lower in shoots than that of Al-tolerant races. In another study (Iannelli *et al.* 2002), *Phragmites* plants exposed to a high concentration of $CdSO_4$ (50 μM) for 21 d accumulated the highest amount of $Cd^{2+}$ in roots followed by leaves. The results are also in agreement with those of Guo *et al*. (2004), who performed a similar study on barley genotypes. On the basis of these results, it may be concluded that the difference in the tolerance to heavy metals (Al and Cd) toxicity among soybean genotypes is associated with uptake and accumulation of these metals mainly by roots.

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