CHRONIC Cd TOXICITY OF BEAN PLANTS CAN BE PARTIALLY REDUCED BY SUPPLY OF AMMONIUM SULPHATE

ХРОНИЧНАТА Cd ТОКСИЧНОСТ ПРИ ФАСУЛЕВИ РАСТЕНИЯ МОЖЕ ДА БЪДЕ ЧАСТИЧНО НАМАЛЕНА ЧРЕЗ ТОРЕНЕ С АМОНИЕВ СУЛФАТ

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ABSTRACT

The effect of ammonium sulphate supply on plant Cd uptake, growth and photosynthesis of bean plants (cv. Limburgse vroege) grown in Cd-contaminated artificial soil was studied. The experiments were performed at controlled conditions in absence or presence of Cd (0 or 50 mg Cd kg^{-1} soil) and with or without supply of ammonium sulphate [0 or 0.687 g $(NH_4)_2SO_4$ kg^{-1}]. Cadmium inhibited both growth and photosynthetic activity of bean plants. The supply of ammonium sulphate had no significant effect on plant Cd uptake and growth inhibition, but to some extend, reduced Cd-induced stress and its negative impact on the photosynthetic performance.

KEY WORDS: cadmium, beans, ammoniun sulphate, oxidative stress, photosynthesis

РЕЗЮМЕ

Изследван е ефектът от приложението на амониевия сулфат върху постъпването на Cd, растежа и фотосинтезата на фасулеви растения (сорт Limburgse vroede), отглеждани върху замърсена с Cd изкуствена почва. Опитите са изведени при контролирани условия в отсъствие или присъствие на Cd (0 или 50 mg Cd kg^{-1} почва) и при торене или без торене с амониев сулфат [0 или 0.687 g $(NH_4)_2SO_4$ kg^{-1} почва]. Установено е, че Cd инхибира растежа и фотосинтетичната активност на фасулевите растения. Приложението на амониев сулфат не оказва съществено влияние върху постъпването на Cd и растежа на растенията, но намалява, в известна степен, проявите на Cd стрес и техния негативен ефект върху фотосинтезата.

КЛЮЧОВИ ДУМИ: кадмий, фасул, амониев сулфат, окислителен стрес, фотосинтеза

INTRODUCTION

Soil contamination by heavy metals is a serious ecological problem all over the world. Generally, industrially contaminated soils consist a mixture of heavy metals (Cd, Zn and Pb), where Cd is the first metal of concern. The entering of Cd into the food chain may provoke both human diseases [15], [9] and well known toxicity effects on plants, animals and microorganisms. Therefore, a significant research effort is now addressing different approaches for sustainable use of metal contaminated soils [21].

In Bulgaria, the so-called "adaptable agriculture" has been adopted on heavy metal-contaminated areas representing cultivation of non-food crops in order to reduce the risks of Cd loading into the food chain as well as to produce a biomass with an added economical value – fibre, oil or fragrance consisiting products [25], [23], [24], [17]. However, some problems related to this approach remain to be solved. For example, the crops grown on metal-contaminated soils often suffer from chronic metal toxicity leading finally to a loss of productivity. This negative effect, to some extend, is due to Cd often showing a higher phytoavailability as compared to the other problematic metals.

In fact, the toxic effects of Cd on plant physiology are well documented [3], [22]. Many sites of its toxic action have been well established e.g. altered enzyme structure and activities [2], [6], disruption in membrane integrity [20], lipid peroxidation [16], etc., but the general picture of the events taking place in Cd-affected cell still remains unclear. Instead of Cd sequestration with phytochelatins (PCs) [14], the good understanding of other aspects of plant defence strategies, for example the scavenging of Cd-induced oxy radicals, could lead to a reduction of Cd phytotoxicity.

Heavy metals, including Cd, can induce essential nutrient deficiency and even decrease concentrations of several macronutrients in plants [18]. Thus, it seems possible to reduce (at least partially) some of the metal-induced negative effects by optimization of plant mineral nutrition. Some data suporting this point of view have been recently accumulated.

A positive effect of sulphur (S) nutrition on Cd detoxification in sugar beet plants has been established [12], [1]. It is known that at sub-optimal S nutrition Cd-exposed plants preferably allocated S to PCs synthesis, which provoked transition S deficiency [8]. Thus, it seems logical by improving plant S nutrition to achieve both - an adequate plant defence against Cd as well as prevention of S deficiency onset. Recently El-Shintinawy [4] has shown that exogenous glutathione (S-rich peptide) counteracted Cd-induced growth retardation effects in soybean seedlings. On the other hand, Panković et al. [11] have shown that the inhibitory effects of Cd on sunflower photosynthesis decreased at optimal nitrogen (N) supply. They found the lowest inhibition of photosynthetic activity by Cd at optimal N supply, when N investment in soluble proteins and Rubisco were at their maximum.

The aim of this study was to test whether the supply of ammonium sulphate, a well known fertilizer consisiting both S and N nutrients, could reduce chronic Cd toxicity in bean plants in laboratory scale experiments. Bean was chosen as a test plant due to its well recognised response to heavy metal stress [19] and a standard artificial soil as a medium in order to avoid the dependance of specific soil properties.

MATERIALS AND METHODS

Plant material, soil treatment and growth conditions

The experiments were conducted in a climate room in Agricultural University of Plovdiv, Bulgaria, at the following conditions: 150 μmol m^{-2} s^{-1} (PAR; photosynthetically active radiation), 14-h photoperiod, day / night temperature of 25±3 / 17±3 °C and relative air himidity of 60±5 / 70±5 %, respectively. Bean plants (cv. Limburgse vroege) were grown in pots filled by an artificial soil.

The artificial soil was prepared following the standard OECD protocol N° 207 [10]. Briefly, for 1 kg artificial soil 100 g sphagnum torf, 700 g quartz sand, 200 g kaoline clay and 4.1 g calcium carbonate were mixed together with 465 ml deionised water. The prepared soil (pH in water 6.5) was left for 5 days and than divided in two parts. One part was treated by water solution of $CdCl_2.H_2O$ giving a final concentration of 50 mg Cd kg^{-1} soil; the remainig part received only deionised water. After a month each soil was additionally split in two parts. One part was amended by ammonium sulphate (AS) in a rate of 0.687 g $(NH_4)_2SO_4$ per kg soil; the other part was not amended. The final experimental design consisted four treatments, namely: 1 (– Cd – AS: control), 2 (– Cd + AS), 3 (+ Cd – AS) and 4 (+ Cd + AS).

Been seeds received a cold treatment (+ 4°C) for a night to break dormancy and to synchronize germination and were imbibited for 6 hours in tap water. Than they were sown in the pots at 2 cm deep and density of four seeds per pot, each containing 500 g soil. The soil water content was adjusted to 40% of its full water holding capacity during germination and increased to 60% thereafter.

Plants were grown for 25 days. After the measurement of leaf gas exchange, plants were harvested and some morphological parameters, such as fresh weight, leaf area, plant height were measured. Samples for the

determination of photosynthetic pigments, peroxidase activity and lipid peroxidation were taken from the first trifoliate leaves of the plants and immediatelly processed. The remaining biomass was dried at 65 °C for 48 hours and used for determination of Cd content plant organs. Samples of both roots and leaves were mineralised at 600 °C for 7 hours. The ash was dissolved in 20% HCl. Content of Cd was determined by inductively coupled plasma emission spectrometry.

Guaiacol peroxidase (GPOD) determination

Leaf samples of 1 g fresh weight were homogenized in 5 ml ice cold extraction buffer (0.1 M Tris-HCl, 1 mM EDTA, 1 mM DTT, pH 7.8) and 4% insoluble polyvinylpyrrolidone. The homogenate was squeezed through a nylon mesh and centrifuged for 10 minutes at 20000 g at 4°C. The supernatants were collected and used for spectrophotometrically measurement of GPOD activity as described by Lagriffoul et al. [6]. The enzyme activity was expressed as mU per g fresh weight, where one unit (U) equals the amount of substrate (μmol) transformed by the enzyme in one minute at 25°C.

Lipid peroxidation

The lipid peroxidation in plant tissues was determined as 2-thiobarbituric acid (TBA) equivalents as described previously [5]. Plant material (0.5 g) was extracted in mortar with sand and 5 ml of 0.5 trichloric acid (TCA). After centrifugation at 20 000 g for 10 min at 20 °C, 1 ml of the supernatant was added to 4 ml of 0.5 % TBA made in 20 % TCA. This solution was boiled in a bath at 95 °C during 30 min and then quickly cooled in ice for 5 min. After centrifugation at 10 000 g for 10 min at 20 °C, the absorbance was measured with Specol 11 spectrophotometer at 532 nm. Correction of non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. The level of lipid peroxidation was expressed as nM g^{-1} FW formed using an extinction coefficient of 155 mM cm^{-1}.

Leaf gas exchange and photosynthetic pigments measurements

Leaf gas exchange (net photosynthetic rate - A, stomatal conductance - g_s and transpiration rate – E) was measured using the first true leaf by an LCA-4 (ADC, England) apparatus under the same conditions excepting the light intensity, which was set up to 400 μmol m^{-2} s^{-1} PAR. Chlorophylls and total carotenoids contents were extracted in acetone, measured spectrophotometrically and calculated according to Lichtenthaler [7].

Statistical analysis

Values obtained were expressed as mean ± SE from 3-5 replicantions. The Student's t-test was used to evaluate the difference between control and other treatments.

RESULTS AND DISCUSSION

The growth parameters (fresh weight, leaf area and plant height) of bean plants grown in absence of Cd were not significantly influenced by ammonium sulphate supply (Table 1). This could be explained by the good mineral status of the artificial soil, which was able to supply enough nutrients during the early growth of bean plants.

Cd-exposed bean plants were distinguished by their inhibited growth and the presence of known toxicity symptoms, such as chlorosis, turning to yellowing of the first trifoliate leaves as well as some browning of the roots [22], [3]. The growth of these plants was signficantly inhibited by Cd. For example, the fresh biomass, leaf area and height of Cd-exposed, but non-amended plants (treatment 3) were diminished by 29, 20 and 43%, respectively, as compared with the control values. In addition, the growth parameters of both amended and non-amended Cd-exposed plants were quite similarsuggesting that the supply of ammonium sulphate did not protect the early plant growth from Cd toxicity. On the other hand, the toxicity symptoms on the trifoliate leaves were much stronger expressed in plants grown on the non-amended soil as compared with those receiving ammonium sulphate.

Obviously, Cd-exposed plans suffered from chronic toxicity and it seemed to be directly related to plant Cd uptake. While Cd concentrations in control plants were within the norm – less than 1 mg kg^{-1} DW in both roots and leaves [22], they sharply increased in Cd-exposed plants being in the range 180-200 mg kg^{-1} in the roots and 22-30 mg kg^{-1} in the leaves. We did not find significant differences in Cd accumulation between the amended and non-amended by ammonium sulphate plants (data not shown). Our results are different from those of Puschenreiter et al. [13] who showed a small increase of Cd uptake by Thlaspi and Amaranthus species due to the soil acidification property of the ammonium sulfate.

The important observation in this study was the different expression of the toxicity symptoms on the first trifoliate leaves of Cd-exposed plants at soil treatments with and without ammonium sulphate. Good illustrations of this effect are the data concerning the photosynthetic pigments content presented in Table 2. The content of Chl. a, Chl. b and total carotenoids in Cd-exposed plants on the non-amended soil was strongly lowered by 67, 70 and 62%, respectively, as compared to the control values. In the same time, the plants at the amended treatment showed lower decrease in the pigments content, in average by 38%. The positive effect of ammonium sulphate on the

Table 1. Fresh weigh (FW; g plant^{-1}), leaf area (LA; cm^2 plant^{-1}) and plant height (PH; cm) of bean plants, grown in with or without Cd contamination as well as ammonium sulphate supply. * - significantly different from the control value at p=0.05 (n=4)

Таблица 1. Свежа надземна маса (FW; g plant^{-1}), листна площ (LA; cm^2 plant^{-1}) и височина (PH; cm) на фасулеви растения, отглеждани в отсъствие или присъствие на Cd и при торене или без торене с амониев сулфат. * - статистически различни от контролната стойност при p=0.05 (n=4)

Treatments	FW	LA	PH
1. – Cd – ammonium sulphate (control)	2.95 ± 0.25	114.5 ± 6.0	14.5 ± 1.5
2. – Cd + ammonium sulphate	2.89 ± 0.17	112.2 ± 8.1	14.2 ± 0.8
3. + Cd – ammonium sulphate	2.09 ± 0.31*	91.8 ± 5.6*	8.4 ± 1.1
4. + Cd + ammonium sulphate	2.14 ± 0.12*	94.5 ± 6.0*	8.5 ± 1.3

Table 2. Chlorophylls a and b and total carotenoids content (all in mg g^{-1} fresh weight) in the first trifoliate leaf of bean plants, grown in with or without Cd contamination as well as ammonium sulphate supply. * - significantly different from the control value at p=0.05 (n=3)

Таблица 2. Съдържание на хлорофил a и b и общи каротеноиди (всички в mg g^{-1} свежа маса) в първия сложен лист на фасулеви растения, отглеждани в отсъствие или присъствие на Cd и при торене или без торене с амониев сулфат. * - статистически различни от контролната стойност при p=0.05 (n=3)

Treatments	Chl. *a*	Chl. *b*	Carotenoids
1. – Cd – ammonium sulphate (control)	1.42 ± 0.10	0.86 ± 0.10	1.56 ± 0.11
2. – Cd + ammonium sulphate	2.10 ± 0.19*	1.01 ± 0.05*	2.22 ± 0.08*
3. + Cd – ammonium sulphate	0.47 ± 0.05*	0.26 ± 0.05*	0.60 ± 0.05*
4. + Cd + ammonium sulphate	0.88 ± 0.07*	0.53 ± 0.08*	0.97 ± 0.11*

photosynthetic pigments content in Cd-exposed plants may be partly explained by the importance of N for chlorophyll biosynthesis as the positive effect of this amendment was also expressed on non Cd-exposed plants (Treatment 2).

Cd may decrease photosynthetic pigments content by inhibition of their biosynthesis as well as oxidative damages [22]. In fact, Cd has a low redox potential and therefore it cannot participate in biological redox reactions, but there exists some evidence that it could perform oxidative related disturbances, including lipid peroxidation [16]. The results, presented in Figure 1, support this standpoint. Both guaiacol peroxidase (GPOD) activity and content of MDA equivalents in the leaves of Cd-exposed plants significantly increased, which in accordance with many authors [3], [4], [6] suggests oxidative damages. GPOD activity and lipid peroxidation were enhanced by 140 and 45%, respectively, as compared with the control values, whereas the respective values in the plants receiving ammonium sulphate were much less - 55 and 24%.

The negative impact of Cd on cell redox status is known and explained by the high affinity of Cd ions to SH-groups of proteins, which may affect their functional properties [20]. When plant cells are not able to maintain low free Cd ions in the cytosol through efficient detoxifying mechanisms, this may lead to a depletion of the cell defence network and as a consequence to oxidative damages to important molecules, including lipids [22]. Obviously, the plants grown on soil amended by ammonium sulphate had a higher capacity to withstand Cd. Having in mind the importance of sulphur nutrition for the cell defence network against Cd through PCs inactivation [8], [14] as well as the role of glutathione for the cell redox status [16], we considered that the ameliorating effect was partly due to improved plant sulphur status allowing better operation of different defence mechanisms.

In the Table 3 data are presented about leaf gas exchange in the first trifoliate leaves of bean plants at different

Table 3. Leaf gas exchange in the first trifoliate leaves of bean plants, grown in with or without Cd contamination as well as ammonium sulphate supply. Photosynthetic rate (A; μmol m^{-2} s^{-1}), transpiration rate (E; mmol m^{-2} s^{-1}), stomatal conductance (gs; mol m^{-2} s^{-1}). * - significantly different from the control value at p=0.05 (n=5)

Таблица 3. Листен газов обмен в в първия сложен лист на фасулеви растения, отглеждани в отсъствие или присъствие на Cd и при торене или без торене с амониев сулфат. Скорост на фотосинтезата, (A; μmol m^{-2} s^{-1}), скорост на транспирацията (E; mmol m^{-2} s^{-1}), устична проводимост (gs; mol m^{-2} s^{-1}). * - статистически различни от контролната стойност при p=0.05 (n=5)

Treatments	A	E	gs
1. – Cd – ammonium sulphate (control)	20.41 ± 2.1	0.62 ± 0.08	0.18 ± 0.02
2. – Cd + ammonium sulphate	21.43 ± 1.8	0.83 ± 0.04*	0.28 ± 0.03*
3. + Cd – ammonium sulphate	8.33 ± 1.1*	0.61 ± 0.05	0.17 ± 0.02
4. + Cd + ammonium sulphate	10.25 ± 0.8*	0.66 ± 0.07	0.20 ± 0.03

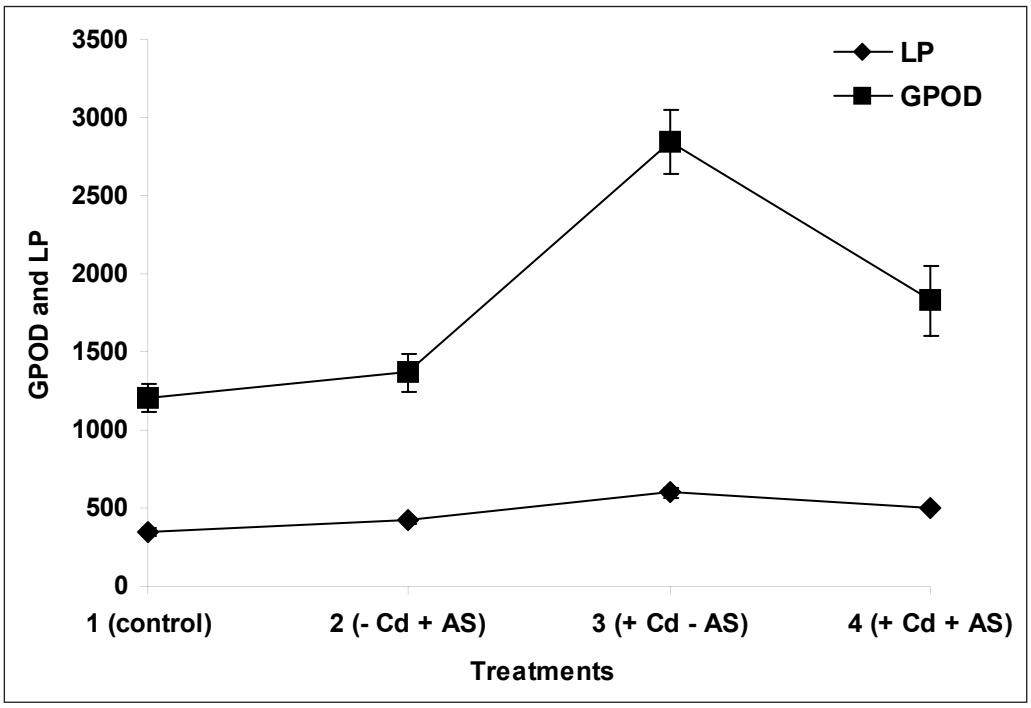

Figure 1. Changes in guaiacol peroxidase GPOD activity (mU g^{-1} FW) and lipid peroxidation (nmol MDA equivalents g^{-1} FW) in the first trifoliate leaves of bean plants grown in absence or presence of Cd and with or without ammonium sulphate (AS) supply. * Significantly different from the control value at p=0.05 (n=3). SE values in LP values are smaller than the symbol.

Фигура 1. Промени в гваякол пероксидазната активност (mU g^{-1} FW) и липидната пероксидация (nmol MDA equivalents g^{-1} FW) в първите сложни листа на фасулеви растения, отглеждани при отсъствие и присъствие на Cd и при торене и без торене с амониев сулфат (AS). * Статистически различни от контролната стойност при p=0.05 (n=3). Стойностите на SE в липидната пероксадация са по-малки от символа.

treatments. Cd-exposed plants showed an about 2 times lower photosynthetic rate than the control plants. Generally, many factors at different structural-functional levels may disturb the photosynthetic process in Cd-exposed plants [22]. However, in this specific case the negative Cd effect was mostly probably due to the lower photosynthetic pigments content, as both stomatal conductance and transpiration rate were not significantly affected by Cd. Again, the effect was less expressed in Cd-exposed plants receiving ammonium sulphate.

Conclusions

The results obtained at these lab-scale experiments showed that chronic Cd phytotoxicity might be partially reduced by the supply of ammonium sulphate. The amendment did not influence plant Cd uptake and early growth inhibition of Cd-exposed bean plants but improved the physiological status of the growing leaves. The plants receiving ammonium sulphate showed better preservation of both photosynthetic pigments content and photosynthetic rate than non-amended ones. They suffered less from Cd-induced stress as indicated by both peroxidase activity and lipid peroxidation. However, the observed positive effect of the ammonium sulphate against Cd phytotoxicity needs to be confirmed in field conditions.

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