

Application of nitrogen nutrition for improving tolerance of soybean seedlings to cadmium

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Abstract

This study investigated the impact of different rate of nitrogen nutrition on soybean seedling tolerance to cadmium treatment. Soil contamination with cadmium has become a serious issue in sustainable agriculture production and food safety. The results indicated common response of soybean seedlings to cadmium treatment such as stunted root growth and increased level of lipid peroxidation. However, a defense reaction was observed under combined seedling treatment with cadmium plus nitrogen. Interaction of cadmium and nitrogen resulted in decreased cadmium uptake by seedling roots that were cultivated at a high nitrogen nutrition level. The obtained data confirms the complexity of plant non-specific defense responses to abiotic stressors and may be used for optimization of plant nitrogen fertilization on cadmium contaminated soil.

Key words: cadmium, defense reactions, *Glycine max*, nitrogen fertilization, soybean.

Abbreviations: AOS, active oxygen species; CAT, catalase; HS, Helrigel solution; LP, lipid peroxidation; TBARS, thiobarbituric acid-reactive substances.

Introduction

Among heavy metals, cadmium (Cd) is considered to have high toxicity to humans and other living organisms (Chen et al. 2007). Recent advances in industry and agriculture have led to an increased level of Cd in the agricultural soil environment. Cadmium enters the soil through various anthropogenic sources including application of phosphate fertilizer, waste water and anthropogenic emissions from power stations, metal industries, urban traffic and cement industries (Alloway, Steinnes 1999; di-Toppi, Gabrielli 1999; Wu et al. 2004; Yang et al. 2004). Due to its high mobility in soil, Cd accumulation in plants grown on Cd-contaminated soils poses a serious threat to human and animal health and can cause both acute and chronic disorders (Jarup 2003). In plants, Cd toxicity can disturb photosynthetic rate, chlorophyll content, stomatal conductance, transpiration rate, relative leaf water content, and other physiological processes, which ultimately reduces plant growth and development (Seregin, Ivanov 2001).

Heavy metals, including Cd, can induce nutrient deficiency and even decrease concentrations of several macronutrients in plants (Siedleska 1995). Thus, it seems possible to mitigate some of the metal-induced negative effects by optimization of plant mineral nutrition. Some

data supporting this point of view have been recently accumulated. It is known that plant nutrients affect the activity and bioavailability of Cd in the soil-plant environment. Several nutrients have many direct as well as indirect effects on Cd availability and toxicity. Indirect effects include dilution of Cd concentration by increased plant biomass and alleviation of physiological stress (Sarwar et al. 2010). Direct impacts result in decreased Cd solubility in soil by promoted precipitation and adsorption, competition between Cd and plant nutrients for the same membrane transporters, and Cd sequestration in vegetative parts avoiding its accumulation in grain / edible parts. For example, phosphate promotes the precipitation of Cd²⁺ (Hong et al. 2010), while ferrous iron (Fe²⁺) competes with Cd²⁺ for the same membrane transporters in plant cells (Vert et al. 2002; Kovacs et al. 2010). Efficiency of nitrate supply recently was shown for phytoextraction of Cd and Zn by the hyperaccumulator *Thlaspi caerulescens*, which showed special relevance for phytoremediation (Xie et al. 2009).

Nitrogen (N) is an essential macronutrient deficient in most soils (Tisdale, Nelson 1975). It is an important component of many structural, genetic and metabolic compounds in plants (Hassan et al. 2005). N as a component of certain molecules can perform a signal function in the

defense reactions of plants against various stresses (Durner, Klessig 1999). Previously, the effect of N deficiency, or application of either nitrate or ammonium on plants and subsequent defense responses have mostly been studied (Polesskaya et al. 2004; Zhao et al. 2005; Chaffei et al. 2008). A limited number of studies are available on the effect of excessive plant nutrition on induced defenses of plants (Lin et al. 2012). It is known that intensive fertilization in modern agriculture often causes surplus of the fertilizer as N can subsequently become a pollutant (Savci 2012). Moreover, application of raised rates of N fertilizer, aimed at increasing the protein amount in crops grain, result in elevated Cd concentration in the grain of plants cultivated on contaminated soil (Wångstrand et al. 2007). N plays an important role in the development of plant stress reaction to various environmental factors. It can also cause soil acidification, which alters heavy metal mobility and availability for plants. We investigated the effect of different rates of N nutrition on soybean seedling tolerance to cadmium, as Cd accumulation in soils of Ukraine recently have become threatening.

Materials and methods

Plant material and growth conditions

Seeds of soybean [*Glycine max* (L.) Merr.], cv. Ustya were surface sterilized and germinated on distilled water. After germination 4-days old seedlings with approximately equal length of seminal roots were cultivated for 48 h on balanced Helrigel nutrient solution (HS) as a control, or with medium and high N content (HS + N 1.2 mM and HS + N 24 mM, respectively). A part of seedlings also were treated with Cd 50 mg L⁻¹ in HS. This concentration is above the average found in the environment (1 mg kg⁻¹ of soil), but evokes significant stress response in soybean roots without lethal effect (Ferreira et al. 2002). NH₄NO₃ and Cd(NO₃)₂ 4H₂O were used for preparation of experimental solutions. In order to prevent acidification of nutrition solutions, pH in all experimental treatments was monitored and adjusted to the optimum level for soybean (pH 5.5) (Legget, Frere 1971).

Analytical procedures

Tolerance of soybean seedlings to stress conditions was estimated by measurement of seminal root growth during 48 h of incubation (Wilkins 1978).

For determination of Cd content in roots, dried plant material (0.2 g) was digested in a mixture of H₂O, HNO₃ and HClO₄ (3:5:1.5, v:v:v). Concentration of Cd in plant material was determined by flame atomic absorption spectroscopy (Perkin-Elmer 1100B Spectrophotometer) (Pulido et al. 1966).

Lipid peroxidation (LP) was determined as thiobarbituric acid reactive substance (TBARS) content in root tissue. Plant material (0.2 g) was ground in 0.1 M Tris-

HCl buffer (pH 7.6). Sample homogenates were prepared in 0.67% thiobarbituric acid and 20% trichloroacetic acid and boiled for 30 min, as described by Heath and Packer (1968). The absorbance of the supernatant was measured at 532 nm. The amount of TBARS was calculated using an extinction coefficient of 155 mM⁻¹ cm⁻¹.

For measurement of catalase (CAT; EC 1.11.1.6) activity, plant material (1 g) was ground in phosphate buffer (pH 7.0). The reaction mixture contained phosphate buffer and enzyme extract. The reaction was initiated by adding 3% H₂O₂. Activity was measured by monitoring changes in optical density in the reaction mixture at 240 nm (extinction coefficient of 39.4 mM⁻¹ cm⁻¹) (Kumar, Knowles 1993).

Statistical analysis

Each experiment was performed at least three times. There were three biological replications for variable determined and 30 seedlings per treatment were used. The data were subjected to analysis of variance (ANOVA) with subsequent Student's t-test or Duncan's multiply range test. Data are expressed as means of replicates ± standard deviation.

Results

Growth response of soybean seminal roots varied between experimental treatments (Fig. 1 A, B). Root growth did not significantly differ between nutrient regimes (0 to 24 mM N). However, there was a tendency for decreased root growth at the highest N dose used (24 mM). Application of Cd to soybean seedlings without additional N or with the highest N dose showed a statistically significant decrease of root fresh mass by 25% (p < 0.01). When a medium dose of N (1.2 mM) was used, the decrease of root growth due to Cd treatment tended to be less. Similar changes were found for root dry mass (data not shown).

Cd accumulation in root tissues of soybean seedlings was about 26% (p < 0.01) lower under high N content in the medium (24 mM), in comparison to Cd + HS + N 1.2 mM and Cd + HS treatments (Fig. 2).

Rate of N availability was not significantly related to LP level as estimated by content of TBARS in soybean roots (Fig. 3). Application of Cd increased LP, with the most intense accumulation of TBARS in root tissues of Cd + HS-treated seedlings (p < 0.05). At higher N doses the effect of Cd on LP was not statistically significant.

CAT activity did not significantly differ in roots of soybean seedlings with different N content, although there was some trend towards a dose-dependent increase of the activity (Fig. 4). In contrast, CAT activity was affected by Cd-treatment. It was found that activity of CAT increased twice (p < 0.001) in response to single Cd treatment (Cd + HS). The same level of CAT activity induction by Cd treatment was found in seedlings treated with Cd + HS + N 24 mM (p < 0.001). Cd and N nutrition had significant effect on enzyme activity in these treatments. Lower CAT

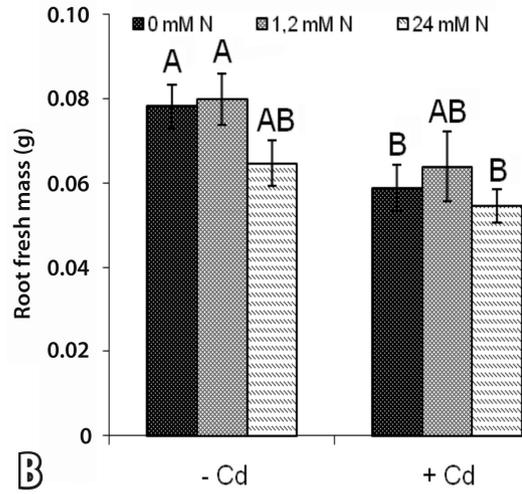
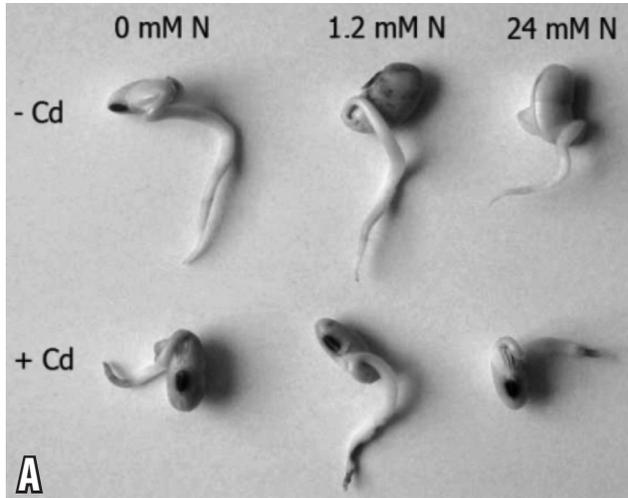


Fig. 1. Growth response of soybean roots to different N application and Cd-treatment (A). Fresh mass of soybean roots under different N application and Cd-treatment (B). Means followed by the same letter were not significantly different at $p < 0.05$ according to Duncan's multiple range test.

activity was observed in soybean seedlings treated with Cd under 1.2 mM N fertilization ($p < 0.05$).

Discussion

The root tip is the most active region of the root for Cd uptake (Lux et al. 2011), and therefore addition of Cd to the nutrient solution resulted in a drastic reduction of plant growth. Even though heavy metal stress induces water uptake disturbances in plants (Seregin, Ivanov 2001) we did not observe any significant difference between changes of fresh and dry mass of soybean seminal roots under the conditions employed. Cd treatment resulted in stunted roots growth and blackening of their apices, which might indicate metal-induced oxidation of phenol compounds – an endogenous factors of growth inhibition, or might be a consequence of oxidative stress (Dietz et al. 1999; Fojtova, Kovařík 2000). Decreased root fresh mass was accompanied by accumulation of Cd in root tissue (Fig. 1, 2). These are common reactions of plants to heavy metal stress (Cao et al. 2007). Even though there is an evidence of symplastic delivery of Cd to xylem (for example, protein-mediated transport of Cd ions), Cd is mostly transported from roots to shoots via an apoplasmic pathway to endoderm and in sublethal concentrations meets difficulty to pass endodermis with its Casparian bands (Seregin, Ivanov 1997). Heavy metal influx can be limited by cell wall impregnation with substances such as suberin, lignin, and callose (Schreiber 1999; Hirano 2006; Franke, Schreiber 2007). In our study, cadmium treatment induced callose localization in the rhizoderma and exoderma cells (Konotop, unpublished data), which is thought to form an additional barrier for cadmium ion uptake and transport to the root stele (Seregin, Ivanov 2001).

Elevated content of Cd in roots of plants exposed to Cd treatment is a direct factor affecting oxidative stress, as

manifested by metabolic disorder and growth inhibition. Cd injury to plants is mainly a consequence of an altered oxidant level, thus causing the occurrence of oxidative stress due to accumulation of active oxygen species (AOS) (Gallego et al. 1996; Chaoui et al. 1997). The extent of oxidative stress in plants can be demonstrated by TBARS concentration, which is a general indicator of LP. Conversely, plant cells may develop defense mechanisms against AOS. One of the protective mechanisms is the enzymatic antioxidant system, which involves the sequential and simultaneous action of a number of enzymes. The results of the current study showed that increased amount of TBARS was associated with free radical formation in seedlings under heavy metal stress, and an increased level of CAT

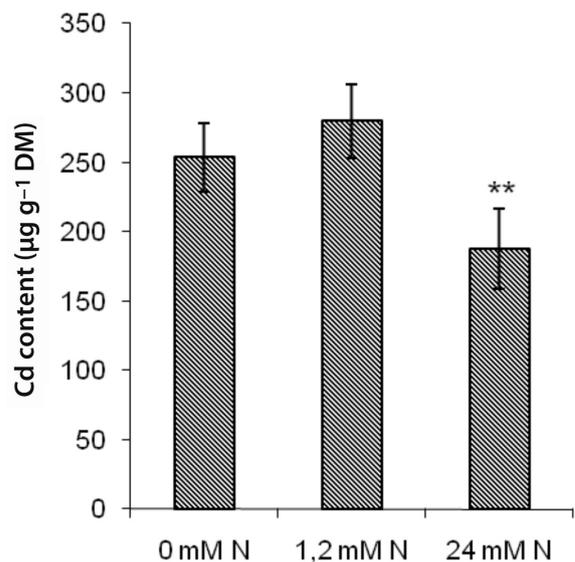


Fig. 2. Content of Cd in soybean roots under different N application and Cd-treatment. Asterisks indicate the significance of difference at the level $p < 0.01$ according to Students t-test.

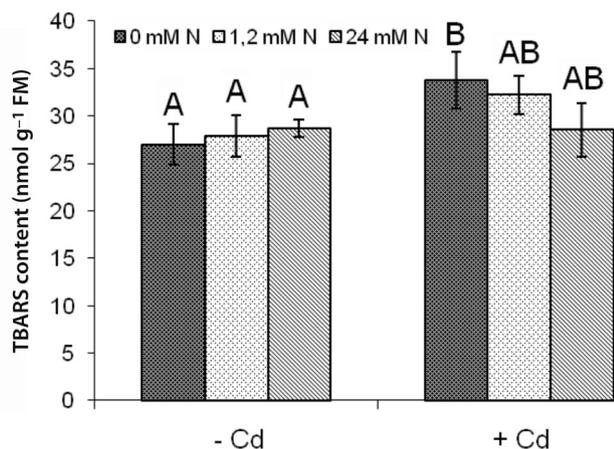


Fig. 3. Content of TBARS in soybean roots under different N application and Cd-treatment. Means followed by the same letter were not significantly different at $p < 0.05$ according to Duncan's multiple range test.

indicated occurrence of a scavenging mechanism (Fig. 3, 4). Dixit et al. (2001) reported that an increased LP level may be a consequence of generation of AOS, which can explain the increase of CAT activity in roots of the Cd-treated seedlings. Rate of N nutrition did not significantly alter content of TBARS in soybean roots. In contrast, N fertilization caused a decreased LP level in *Agrostis palustris* under water deficiency (Saneoka et al. 2004). The results of Zhang et al. (2007) suggest that addition of N increases production and drought tolerance in crops by enhancing antioxidant enzyme activity and reducing the LP level.

It is known that concentration of Cd ions in the root is influenced by the Cd content in substrate, the phytoavailability of Cd and the duration of exposure (Lux et al. 2011). In our experiment it was found that the effect of Cd was apparently dependent on the N regime applied. That was shown by decreased root growth of seedlings under Cd + N 24 mM application. Surprisingly, a medium dose of N slightly enhanced but excess of N significantly suppressed the uptake of Cd in soybean roots. These results are consistent with data of Singh et al. (1988), who observed a similar effect of high dose of N in nutrient solution on Cd accumulation in *Lactuca sativa*. Some authors explain the decreased Cd uptake under N by a "diluting effect", resulting from increased biomass production (Sarwar et al. 2010). However, this can not explain the obtained result, as root growth was reduced (Fig. 1, 2). It is well known that N is taken up by plants both in the form of ammonium (NH_4^+) and nitrate (NO_3^-), and in plants NO_3^- is also reduced to NH_4^+ for assimilation into plant organic N (Jalloh et al. 2009). For improved crop growth, a combination of ammonium (NH_4^+) and nitrate (NO_3^-) sources is preferred (Fageria et al. 2009). NH_4NO_3 , which was used in our experiment, is a physiologically acidic fertilizer. The major effect on increased Cd uptake from soil is likely due to NH_4^+ , via a decrease in soil pH (Sarwar

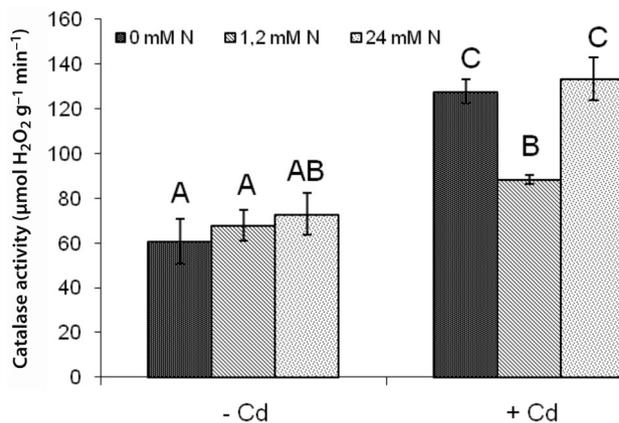


Fig. 4. Catalase activity in soybean roots under different N application and Cd-treatment. Means followed by the same letter were not significantly different at $p < 0.05$ according to Duncan's multiple range test.

et al. 2010). Conversely Zaccheo et al. (2006) reported that increased uptake of NH_4^+ reduces Cd uptake in cells of sunflower. Enhanced acquisition of NH_4^+ in root cells occurs as a result of cell membrane potential depolarization caused by ammonium ions of fertilizer. Similarly, Nasraoui-Hajaji et al. (2012) reported that an ammonium regime strongly protects *Solanum lycopersicum* from Cd toxicity through reduced Cd uptake, LP and improved AOS scavenging activity of antioxidant enzymes. Kováčik et al. (2011) observed decreased Cd accumulation in roots in N-deficient conditions. The authors explain reduced Cd uptake by strong accumulation of phenolic metabolites and nitrate elimination. Due to nitrification process, which occurs after adding N to soil, pH of soil decreases, thus leading to significant increasing of heavy metal solubility in soil and hence decreased heavy metal ion adsorption (Williams 1967).

The results of this study showed that the oxidative stress caused by Cd and 24 mM N was below the level of irreversible damage, thus allowing plants to adapt it actively by increasing CAT activity, indirectly leading to decreased TBARS accumulation. Enzyme activity under Cd treatment and 1.2 mM N was affected in the opposite direction, indicating complexity of plant defense response. We suppose that signaling pathways and cross-talk interaction that occur between the studied stressors could contribute to the defense response of seedlings. It is known that the level of N nutrition affects the endogenous giberellin and jasmonic acid contents (Jang et al. 2008) which in turn regulate plant antioxidant system (in particular enzymes CAT and superoxide dismutase) under abiotic stress (Sedghi et al. 2012). However, further research is needed to test whether this can explain the joint action of Cd and N nutrition on soybean. Extra N supply alters the metabolism of plant nitrogenous compounds that perform signal functions (Sakakibara 2003). It was found

that Cd-supply caused an increased partitioning of nitrate in roots of *Arabidopsis*, as a result of induction of NRT1.8 expression and repression of NRT1.5, specific transporters which are responsible for loading/unloading of nitrate into/from the xylem sap respectively. As NRT1.5 and NRT1.8 mediate opposite transport processes, this suggests that Cd²⁺ triggers coordinated and inverse regulation of the two genes to prevent nitrate loading into the xylem sap. Retention of nitrate in the roots serves as a signal to enhance nitrate assimilation in these organs. Since glutathione and phytochelatins are N-containing molecules, this could lead to increased synthesis of these chelators to detoxify Cd ions (Gojon, Gaymard 2010). Although some of the key factors involved in the transduction of N signals have been identified, the whole picture remains unclear.

From present study it was concluded that N supply can be used as a means of decreasing Cd toxicity to soybean seedlings, due to decreased accumulation and development of oxidative processes in roots. However further research is still needed to fully understand mechanisms of interaction between N and Cd in plants and to determine the optimum N rate for specific plant species and growth conditions in order to successfully minimize Cd toxicity and at the same time to avoid nitrate contamination of the environment.

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