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Differential effect of Cd^{2+} and Ni^{2+} on amino acid metabolism in soybean seedlings

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Abstract

In 10-d-old soybean seedlings, the growth of roots and shoots was significantly inhibited at 50 and 100 μM and more Cd^{2+}, respectively, and by 50 μM or more Ni^{2+}. Although total protein content of roots exposed to 200 μM Cd^{2+} or Ni^{2+} was similarly decreased compared to the control, the activity of nitrate reductase was much more inhibited by Cd^{2+}. Ni^{2+}-treatment (200 μM) induced an accumulation of all free amino acids in roots associated with a decrease in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities reflecting the accumulation of both alanine and aspartic acid, respectively. Cd^{2+}-treatment (200 μM) decreased the amount of all free amino acids. In addition, cysteine which is the main amino acid consisting the phytochelatin complexes constituted about 17.5 % of total free amino acids. The activities of both ALT and AST in Cd^{2+}-treated roots were higher than in Ni^{2+}-treated roots suggesting higher conversion of alanine and aspartate to pyruvate and oxaloacetate. Primary leaves excised from either Cd^{2+} or Ni^{2+}-treated seedlings showed similar pattern of enzyme activities as roots.

Additional key words: alanine aminotransferase, aspartate aminotransferase, cysteine, *Glycine max*, heavy metals, HPLC, nitrate assimilation.

Introduction

Cadmium and nickel have been shown to interfere with plant growth at the cellular, organ and organismal levels (Sheoran *et al.* 1990, Bishnoi *et al.* 1993). Cadmium can be readily taken up and accumulated by vascular plants (Prasad 1995), including soybean (Cataldo *et al.* 1983, Kawashima *et al.* 1991), and has negative effects on plant growth, metabolism and enzyme activity even at low concentrations (Van Assche and Clijsters 1990). Low concentrations of nickel are necessary in nitrogen metabolism and germination of plants (Dalton *et al.* 1985, Krogmeier *et al.* 1991, Brown *et al.* 1990). However, high concentrations of nickel inhibited enzymatic activity (Mattioni *et al.* 1997), growth, metabolism (Baccouch *et al.* 1998), and mineral nutrition (Barcelo and Poschenrieder 1990). During the period of environmental stress, plants enhance the synthesis of a number of products including alanine (Good and Crosby 1989) which is converted, reversibly, to pyruvate by alanine aminotransferase (Lillo 1984). Also aspartate aminotransferase that catalyzes the reaction of aspartate to oxaloacetate has been detected in many plant species (Griffith and Vance 1989). Metal chelating through synthesis of phytochelatin complexes seems to be the most prevalent mechanism enabling the plant cell to tolerate the presence of heavy metals (Robinson *et al.* 1993, Florijn *et al.* 1993, Becher and Höfner 1994).

The present investigation aims to monitor the changes in amino acid metabolism in soybean seedlings upon the exposure to potentially toxic concentrations of Cd^{2+} and Ni^{2+}. Since the regulation of nitrogen assimilation and amino acid metabolism is interconnected, the activities of nitrate reductase, alanine aminotransferase and aspartate aminotransferase were measured to test the ability of soybean seedlings to tolerate both heavy metals.

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Abbreviations: ALT - alanine aminotransferase; AST - aspartate aminotransferase.
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Materials and methods

Plants: Soybean (*Glycine max* L.) seeds were surface sterilized with 2.5 % sodium hypochlorite for 20 min and washed thoroughly with distilled water. The seeds were germinated in Petri dishes moistened with distilled H_2O at 37 °C in dark. After 24 h, uniformly germinated seeds were selected and transferred to Petri dishes (9 cm) containing two sheets of *Whatman No. 1* filter paper moistened with 10 cm^3 of distilled water. On the 3rd day, 3 cm^3 of distilled water or test solutions (different concentrations of either $CdCl_2$ or $NiCl_2$) was added to each Petri dish. After 10 d, roots and shoots were excised at intervals indicated for the different measurements.

Extraction and determination of proteins and free amino acids: Total protein content was determined according to Bradford (1976). Amino acids were analyzed by HPLC according to the method of Weibull *et al.* (1990). The free amino acids were assayed after deprotenization of the dried tissues (0.3 g, prepared from 5-cm long root tips) with 5 % sulphosalicylic acid. The supernatant was filtered through *Millipore* membrane filter (0.45 μm). The filtrate was derivatized (0.025 cm^3) and injected according to the *Pico-Tag* procedure for total amino acids.

Amino acid derivatization: The filtered sample (0.025 cm^3) in 6 × 50 mm tube was placed into drying vessel and dried in *Waters Pico-Tag* workstation for 10 - 15 min. The sample was dried again in the workstation with 0.03 cm^3 of the drying solution containing 0.2 cm^3 methanol, 0.2 cm^3 0.2 M sodium acetate and 0.1 cm^3 triethylamine. 0.03 cm^3 of the freshly prepared derivatization reagents (0.35 cm^3 methanol, 0.05 cm^3 HPLC grade water, 0.05 cm^3 triethylamine and 0.05 cm^3 phenylisothiocyanate) was added to the tube contents and allowed to react for 20 min. 0.03 cm^3 of HPLC grade methanol was added to the tube and left for 15 min; 0.1 cm^3 of the sample was transferred to the injection vials. The standard amino acids solutions were treated typically as the sample.

Analysis of amino acids: The apparatus used is *Spectra-Physics Analytical A0099-600* (Waters, USA) consisting of spectra focus optical scanning detector, spectra system UV 2000 detector and an ultrasphere C_{18} *Beckman* column (4.6 × 150 mm, particle size 5 μm). A gradient of *Pico-Tag* solvents at 40 °C and flow rate of 0.001 cm^3 min^{-1} were adjusted for the analysis process. The calibration was carried out by two injections of the standard amino acids and the retention times were determined. The separated *Pico-Tag* amino acids were detected at 254 nm.

Preparation of extracts and enzyme assays: Extracts were prepared from 5 cm long root tips or from primary leaves randomly chosen from soybean seedlings. The different plant parts were rinsed in distilled water, weighed (0.02 g) and ground with sand in a mortar and pestle in phosphate buffer (pH 7.4). The brei was centrifuged for 15 min. The supernatant was assayed for enzyme activities. Nitrate reductase (E C.1.6.6.1) activity was measured following the method of Neyra and Hageman, (1975). The decrease in absorbance was monitored at 340 nm. Activities of alanine amino-transferase (ALT, EC.2.6.1.2) and aspartate amino-transferase (AST, EC. 2.6.1.1) were measured following the method of Bergmeyer *et al.* (1974) using 2,4-dinitro-phenyl hydrazine as colour reagent. 0.2 cm^3 homogenate equivalent to 0.02 g plant tissues (roots or primary leaves) was incubated in 1 cm^3 substrate-phosphate buffer (pH 7.4) mixture containing 0.2 M phosphate buffer and 0.002 M 2-oxoglutarate with 0.2 M DL-alanine included for ALT and AST, respectively. The incubation time was 30 min for ALT and 60 min for AST at 37 °C. Pyruvate or oxaloacetate reacts with 2 M 2,4-dinitrophenyl-hydrazine and the colour intensity of the hydrazone produced in alkaline medium was measured. Pyruvate solution (0.6 mmol cm^{-3}) treated in the same manner was used as standard and read at 525 nm.

Results and discussion

Fresh and dry masses of seedlings exposed to different concentrations of Cd^{2+} or Ni^{2+} for different intervals were reduced. Root and shoot masses decreased after 10 d treatment with 50 M Cd^{2+} or more and 100 μM or more Cd^{2+}, respectively, and both with 50 M Ni^{2+} or more (Fig. 1). The reduction was a concentration dependent and increased with the length of the exposure period. The differential effect of Cd^{2+}; and not of Ni^{2+}, on root and shoot growth reduction could be explained by the fact that about 98 % of the accumulated Cd^{2+} is retained by soybean roots (Cataldo *et al.* 1983). Roots of seedlings treated with either Cd^{2+} or Ni^{2+} turned brownish 1 d after the beginning of exposure, were less flexible and broken more easily than those of controls. In adition, the primary leaves showed chlorosis progressing from the tip backward followed by necrosis at the tip on day 7 or 8 of metal treatment.

The total protein contents in Cd^{2+} and Ni^{2+} treated

roots were similarly reduced regardless of the type of metal (Table 1). On the other hand, Cd^{2+} inhibited the activity of nitrate reductase (NR) by about 54 % while Ni^{2+} only by about 11 % compared to the control. Thus, Cd^{2+} was more effective than Ni^{2+} in inhibiting the activity of NR as shown earlier by Mathys (1975) using *Silene vulgaris*. The inhibition of NR activity by Cd^{2+} was in agreement with that observed by Nussbaum *et al.* (1988) in maize seedlings. Ferrettei *et al.* (1993) showed that Cd^{2+} also altered the enzymes of sulfate and nitrate assimilation in maize. In addition, the changes in enzyme activity and in the mobilization of food reserves upon the exposure of germinating pigeon pea seedlings to Cd^{2+} or Ni^{2+} were mentioned by Bishnoi *et al.* (1993). The

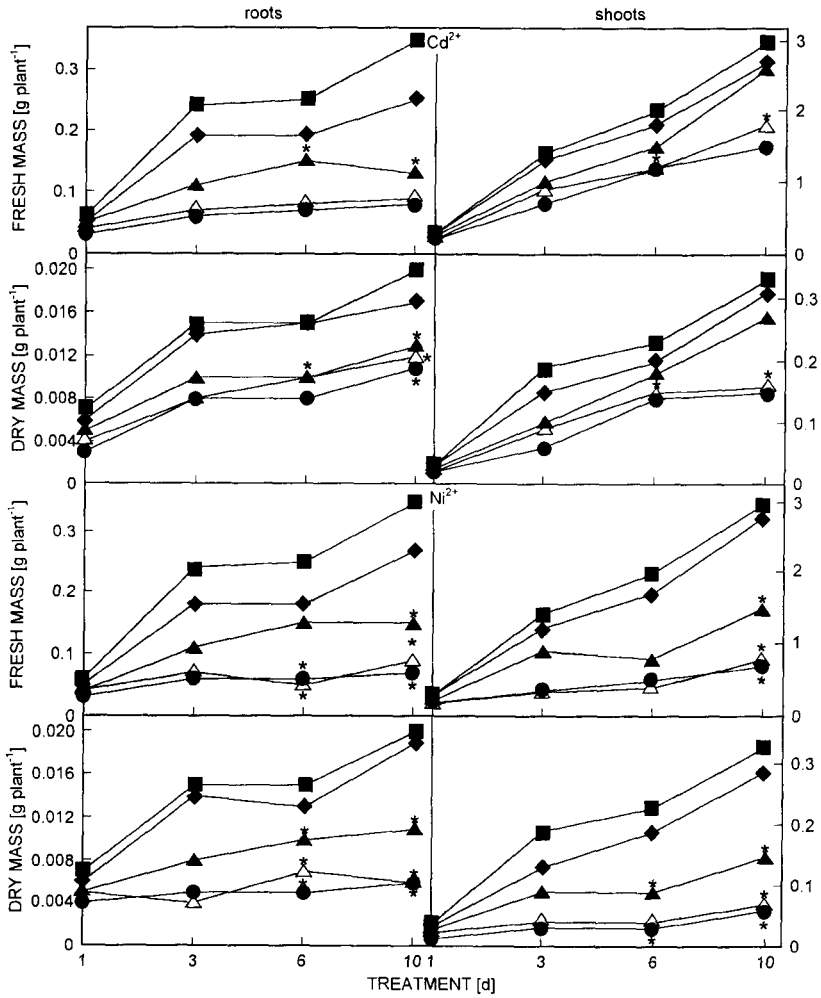

Fig. 1. Changes in fresh and dry masses of roots and shoots of soybean seedlings treated by 0 (*squares*), 5 (*rhombs*), 50 (*closed triagles*), 100 (*open triangles*), and 200 (*circles*) μM Cd^{2+} (*above*) or Ni^{2+}(*below*). Mean values from 5 plants are presented, values followed by an *asterisk* are different from control at $P \leq 0.01$.

Table 1. Protein content [mg g^{-1}(d.m.)] and nitrate reductase (NR) activity [μmol g^{-1}(protein) s^{-1}] in soybean roots treated for 10 d with 200 μM Cd^{2+} or 200 μM Ni^{2+}. Means ± SE, $n = 5$.

	Control	Cd^{2+}	Ni^{2+}
Soluble	36.12 ± 0.01	28.50 ± 0.01	28.22 ± 0.03
Insoluble	17.21 ± 0.02	13.51 ± 0.01	12.33 ± 0.01
Total protein	53.33 ± 0.01	42.01 ± 0.02	40.55 ± 0.02
NR	12.90 ± 0.01	5.94 ± 0.01	11.52 ± 0.02

Table 2. Free amino acids [μmol g^{-1}(d. m.)] in roots treated for 10 d with 200 μM Cd^{2+} or 200 μM Ni^{2+}. Mean values from three experiments ± SE are presented.

Amino acid	Control	Cd^{2+}	Ni^{2+}
Aspartic acid	1.66 ± 0.02	0.92 ± 0.08	4.43 ± 0.05
Glutamic acid	1.67 ± 0.05	0.67 ± 0.01	2.99 ± 0.04
Serine	3.37 ± 0.03	1.18 ± 0.06	24.84 ± 0.04
Glycine	0.46 ± 0.01	0.17 ± 0.01	2.90 ± 0.05
Histidine	1.13 ± 0.02	0.71 ± 0.05	8.19 ± 0.06
Arginine	0.88 ± 0.01	0.32 ± 0.05	5.55 ± 0.08
Therionine	0.89 ± 0.01	0.58 ± 0.04	4.89 ± 0.01
Alanine	0.20 ± 0.05	0.24 ± 0.01	0.83 ± 0.07
Proline	1.03 ± 0.05	0.02 ± 0.02	1.88 ± 0.09
Tyrosine	0.57 ± 0.02	0.75 ± 0.02	2.95 ± 0.01
Valine	0.78 ± 0.08	0.10 ± 0.04	4.33 ± 0.02
Methionone	0.07 ± 0.01	0.12 ± 0.02	0.03 ± 0.02
Cysteine	0.25 ± 0.01	1.27 ± 0.01	2.28 ± 0.01
Isoleucine	0.002 ± 0.001	0.01 ± 0.01	0.26 ± 0.01
Leucine	0.05 ± 0.01	nd.	0.04 ± 0.01
Phenylalanine	0.10 ± 0.01	0.05 ± 0.01	1.45 ± 0.01
Lysine	0.79 ± 0.05	0.17 ± 0.01	1.44 ± 0.01
Total	14.8	7.10	69.88

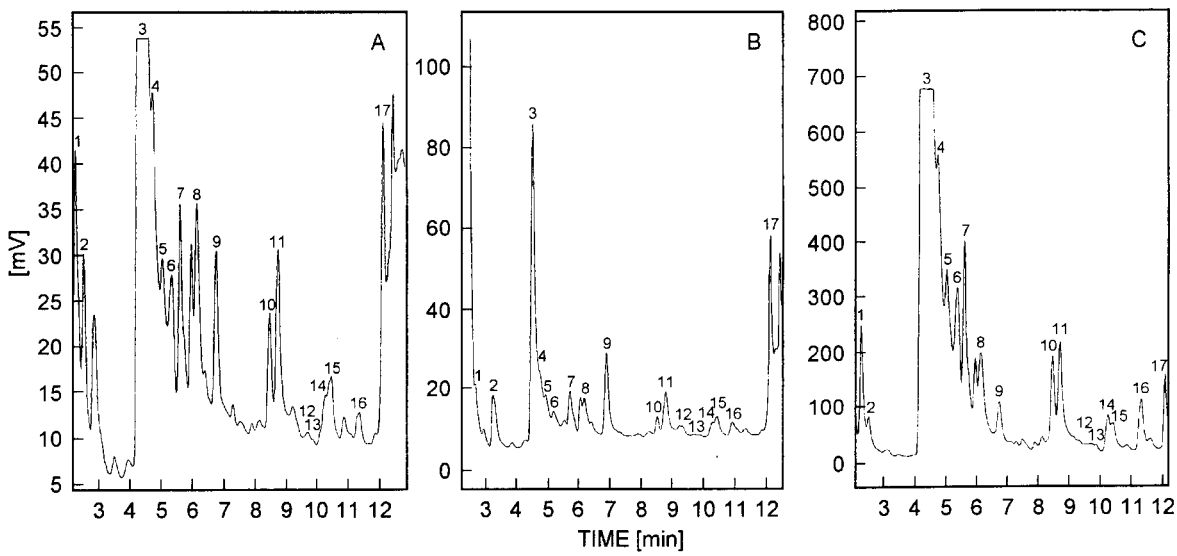

Fig. 2. HPLC analysis of amino acids extracted from soybean control roots (*A*) and roots exposed for 10 d to 200 μM Cd^{2+} (*B*) or 200 μM Ni^{2+} (*C*). Peaks identified by amino acid analysis of relative retention time are: 1 - aspartic acid, 2 - glutamic acid, 3 - serine, 4 - glycine, 5 - histidine, 6 - arginine, 7 - therionine, 8 - alanine, 9 - proline, 10 - tyrosine, 11 - valine, 12 - methionine, 13 - cysteine, 14 - isoleucine, 15 - leucine, 16 - phenylalanine, 17 - lysine.

decrease in NR activity induced by Cd^{2+}-treatment was connected with inhibition of NO_3 uptake and translocation in bean and tomato (Quariti *et al.* 1997) and in peas (Hernández *et al.* 1997).

Pattern, distribution and concentration of free amino acids extracted from control soybean roots (Fig. 2*A*) and those exposed for 10 d to either 200 μM Cd^{2+} (Fig. 2*B*) or 200 μM Ni^{2+} (Fig. 2*C*) were determined using HPLC. The amount of total free amino acids after Cd^{2+} exposure was half of control (Table 2) which was in agreement with those observed by Costa and Spitz (1997) in *Lupinus albus* and in lettuce (Costa and Morel 1993). In contrast, Ni^{2+} exposure increased total free amino acids by about 4.7 fold which was in agreement with that observed by Krämer *et al.* (1996).

The two cations had a different effect on the amino

acids composition in the root tissues. In Cd^{2+}-treated roots, cysteine was the most predominant ones among free amino acids (17.4 %). Thus, Cd^{2+} enhanced the conversion of serine to cysteine. The high concentration cysteine could be explained by the fact that several plants have the ability to synthesize phytochelatin cysteine-rich complexes that are known to bind Cd^{2+} and occur mainly in roots (Steffens 1990, Florijn *et al.* 1993). In addition, Nussbaum *et al.* (1988) explained the increased activity of sulphate assimilating enzymes in Cd^{2+}-treated *Zea mays* roots by the increased demand for cysteine production needed for phytochelatin synthesis. Moreover, Tukendorf and Rauser (1990) compared the influence of excess Cd^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} on the synthesis of phytochelatin complexes in roots of maize seedlings and concluded that phytochelatin synthesis was specific for Cd^{2+}-treatment.

A remarkable accumulation of aspartic acid and alanine in Ni^{2+}-treated roots was observed (Table 2). For a better understanding of the possible involvement of Cd^{2+} and Ni^{2+} in the accumulation of the two mentioned amino acids, the activity of aminotransferases was measured (Table 3). The activity of alanine amino-transferase (ALT) that converts, reversibly, alanine to pyruvate and aspartate aminotransferase (AST) in 10 d old roots exposed to 200 μM Cd^{2+} or Ni^{2+} decreased more with Ni^{2+}.

Table 3. Alanine aminotransferase (ALT) and aspartate aminotranferase (AST) activities [μmol g^{-1}(protein) s^{-1}] in roots and leaves of soybean seedlings treated for 10 d with 200 μM Cd^{2+} or 200 μM Ni^{2+}. Means from five experiments ± SE.

	Control	Cd^{2+}	Ni^{2+}
ALT roots	13.20 ± 0.04	12.01 ± 0.10	10.56 ± 0.10
ALT leaves	17.40 ± 0.04	17.10 ± 0.25	16.53 ± 0.18
AST roots	24.00 ± 0.10	23.72 ± 0.15	18.72 ± 0.04
AST leaves	41.40 ± 0.10	39.33 ± 0.08	23.20 ± 0.09

In primary leaves, the same pattern of inhibition of both enzyme activities was observed. Good and Crosby (1989) recommended that ALT and dehydrogenases activities are parallel, thus, the activity of dehydrogenases is predicted to be lowered in Ni^{2+}-treated roots compared to Cd^{2+}-treated once. The significant alterations in both enzyme activities detected in soybean seedlings may be involved in the general response of higher plants to the uptake of toxic amounts of metals such as Cd^{2+} and Ni^{2+} as discussed by Sheoran *et al.* (1990). This alteration may be related to the kind and concentration of metals as indicated by Mattioni *et al.* (1997). The alteration in both ALT and AST activities may be due to the dramatic inhibition of NO_3 uptake, which is the most common nitrogen source, used by higher plants (*e.g.*, Hernández *et al.* 1997, Quariti *et al.* 1997).

If the activity of NR is regulated according to the need for amino acids for peptide synthesis, the following changes should be expected. The Cd^{2+}-treated roots producing phytochelatin instead of proteins should show a decrease in NR activity accompanied with an increase in cysteine level to synthesize cysteine-rich peptides which is a known mechanism of detoxification (Ernst *et al.* 1992, Florijn *et al.* 1993). The more pronounced inhibition in root growth under nickel treatment could be explained as follows: Ni^{2+} decreased NR, ALT and AST activities and increased total free amino acid content. The accumulation of certain amino acids in cytosol or vacuoles might lead to a damage of the plant cells as they are not involved in further metabolism (Miflin and Lea 1982, Kneer and Zenk 1992).

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