Effect of manganese on cadmium toxicity in maize seedlings

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ABSTRACT

The interaction of manganese with cadmium (Cd) toxicity was studied on maize plants grown in hydroponics. Manganese supplied as $MnSO_4$ clearly alleviated the toxic effect of cadmium on the root growth of maize seedlings. The magnitude of alleviation was dose dependant and total abolition of 10μM Cd toxicity on root growth was observed at Mn/Cd ratio of 20:1. The 12 h pre-treatment with 10μM Cd was generally toxic for nitrate uptake and reduction (both determined in Cd-free media). The beneficial effect of 100μM Mn on this toxicity was confirmed for the low-affinity nitrate uptake system, but on the other hand, Mn alone seems to be slightly toxic for high affinity nitrate uptake system and on the nitrate reductase activity.

Keywords: cadmium; manganese; membrane potential; nitrate uptake; nitrate reduction; maize roots

Cadmium (Cd) is a non-essential and toxic element without any known metabolic significance. The co-existence of Cd with other essential and non-essential heavy metals in the ecosystem can lead to various synergic and antagonistic interactions in their uptake and tissue content. Cadmium addition significantly reduced the Zn and Mn level in roots and shoots of barley (Wu et al. 2003) and durum wheat (Jalil et al. 1994). Similarly, the uptake and toxicity of Cd can be moderated significantly in the presence of excess amounts of certain essential metal nutrients like Zn, Ca, Fe, Cu, Mn (Das et al. 1997, Aravind and Prasad 2003), or non-essential Ti, as was reported recently (Leskó et al. 2002).

Manganese (Mn) is one of the essential heavy metals. The increased level of Mn reduced the uptake of Cd from nutrient medium, which resulted in lower accumulation of Cd in plant tissues (Jarvis et al. 1976, Baszynski et al. 1980). The decrease of plant Cd level after addition of Mn, and vice versa, might be explained by competition for the same membrane-transporters. Recently, due to an effort in sequencing of the whole plant genomes, many multispecific metal transporters have been identified and described. The NRAMP and IRT1 genes encode integral membrane proteins that can transport Mn as well as Cd among other metals (Rogers et al. 2000, Thomine et al. 2003, Cohen et al. 2004). The tobacco plants overexpressing the *Arabidopsis* CAX2 gene for Ca transporter, accumulate significantly higher levels of Mn and Cd in tonoplast (Hirschi et al. 2000). The high Mn level in white lupine *(Lupinus albus* L.), a Mn hyperacumulator, could contribute to the alleviation of negative effects of Cd, especially on the photosynthesis (Zornoza et al. 2002). A higher Mn accumulation in chloroplast when Cd is present in the growth medium was also found in lettuce (*Lactuca sativa* L., Ramos et al. 2002). Moreover, the partial restoration of chloroplast structure damaged by Cd treatment was found after addition of Mn (Baszyński et al. 1980).

The nitrogen nutrition is one of the most important metabolic processes in plants. Several authors found that nitrate uptake and assimilation is negatively affected by the presence of Cd in the growing medium. In particular, nitrate uptake decreased after Cd treatment in pea (Hernández et al. 1997), wheat (Stolt and Oscarson 2002) or barley (Boussana et al. 1999). Similarly, Cd inhibited the activity of nitrate reductase and nitrite reductase, the enzymes involved in nitrate reduction (Chugh et al. 1992, Ouariti et al. 1997) as well as glutamine synthetase, the enzyme involved in nitrogen assimilation (Chiraz et al. 2003).

The aim of the present study was to investigate the alternations in the growth, nitrate uptake and reduction in maize plants grown in the presence of different combinations of cadmium and manganese in culture medium. The possible positive effect of Mn on Cd toxicity is discussed.

MATERIAL AND METHODS

Plant material and growth conditions. Maize (*Zea mays* L. cv. Tina) seeds were placed in filter papers rolls soaked with distilled water and germinated in the dark at 25°C and 98% RH. Three-day-old seedlings with 5–6 cm long primary seminal roots were used for growth experiments (see below), or transferred into N-free, half strength Hoagland's solution and kept in a growth chamber E8 (Conviron, Canada) under controlled conditions (24°C, 70% RH, 14/10 h light/dark cycle and photosynthetic quantum flux density 180 µmol/m^2/s). Nutrient solution was continuously aerated and changed 2 times a week for a fresh one. 14-day-old seedlings were used for uptake, nitrogen content and nitrate reductase activity measurements after 12 h pre-treatment with induction solution containing 0.2mM (for uptake) or 2mM (for nitrate reductase activity) KNO_3, 0.5mM $CaSO_4$ (control); and 10µM $CdCl_2$ with different concentrations of $MnSO_4$ (0–1mM), (Cd and Mn treated plants). For root growth determination, three-day-old seedlings were selected for uniformity and transferred to glass pots containing 0.5mM $CaSO_4$, and different concentrations of Cd (0–1mM), supplied as $CdCl_2$ (pH 5.0). To screen the effect of Mn on root growth under Cd stress, the seedlings were treated by 10µM Cd, in combination with 1µM to 1mM $MnSO_4$. All seedlings were left in dark at 24°C. The growth rate of roots was calculated as an increase of root length between final and initial root lengths and expressed in cm/day.

Nitrate uptake and nitrate content. Nitrate uptake was calculated by following depletion from the uptake solution containing 0.5mM $CaSO_4$ and 0.2 or 2mM KNO_3 (for high and low affinity uptake systems, respectively). Concentrations of NO_3^- were determined spectrophotometrically (Braun-Systematic, Methodenblatt N60) and related to the root fresh weight. The minimal concentration of the solution was 0.05mM for high affinity uptake conditions and 1mM for low affinity uptake conditions at the end of experiment. Determinations of nitrate content in root crude extracts from 4 cm long apical segments were performed as described by Pajuelo et al. (2002). 200 µl of 5% (w/v) salicylic acid dissolved in 96% (w/v) sulphuric acid was added to aliquots of 50 µl from the root crude extracts (5 ml/g FW) and left to react for 20 min. 4.75 ml of 2M NaOH was added to the reaction mixtures and then the absorbance was read at 405 nm after cooling. A calibration curve of known amounts of nitrate dissolved in the standard extraction buffer was used for analytical determinations. Blanks were set up without salicylic acid.

Cadmium and manganese content. The cadmium and manganese content was determined in 5 cm long root segment of the maize seedling treated the same way as for nitrate uptake measurements. The dry roots were digested in teflone autoclave in nitric acid for 4 h at 160°C, and dried in fluoric acid. The dry matter was digerated in nitric acid. The Cd and Mn content were determined by atomic absorption spectrometry at 228.8 nm for Cd and 279.5 nm for Mn.

Measurement of membrane potential. Three-day-old maize seedlings after 24 h pre-incubation in solution containing 1mM $CaSO_4$, 0.2mM KNO_3 and combinations of 10µM $CdCl_2$ and 10–200µM $MnSO_4$, respectively, were attached to a Plexiglas holder and mounted in a vertical 5 cm^3 cuvette perfused with solution containing 1mM $CaSO_4$ (pH 5.5). Nitrate was added by replacing the 1mM $CaSO_4$ by 1mM $Ca(NO_3)_2$, Measurements of the membrane potential (Em) were carried out at 22°C, using standard microelectrode techniques (Pavlovkin et al. 1993) in the outermost layer of the root cortex cells 3–5 mm behind the root cap.

Nitrate reductase assay. For nitrate reductase activity, 4 cm tips or basis of plant roots were quickly frozen in liquid nitrogen, homogenised and then kept at –80°C until use. Root material (0.1 g) was ground in a mortar at 4°C and resuspended in extraction buffer (0.6 ml). Extraction buffer contained: 100mM HEPES-KOH (pH 7.6), 10mM EDTA, 1mM dithiothreitol (DTT), 1mM phenylmethylsulphonylfluoride (PMSF), 3% (w/v) bovine serum albumin (BSA), and 0.1% (v/v) Triton X-100. The homogenate was centrifuged for 15 min at 15 000 × *g* and insoluble material was removed. The clear supernatants were immediately assayed for NR activity (Pajuelo et al. 2002). The NRA_{tot} assay was carried out as follows: 500 µl final volume reaction mixtures were placed in 1.5 ml Eppendorf tubes containing: 250 µl of assay buffer (100mM HEPES-KOH pH 7.6, 10mM EDTA and 20µM FAD), 50 µl 100mM KNO_3; 50 µl of extraction buffer and 100 µl root crude extract. The reaction was started by addition of 50 µl 0.7mM NADH and carried out at 30°C for 30 min. The NRA_{act} determination was performed by the same way, except that 10mM EDTA was replaced by 10mM $MgCl_2$ both in extraction and assay buffer. Nitrite formation was measured as described elsewhere (De la Rosa et al. 1982), using sulphanilamide and N-(1-naphtyl)-ethylenediamine dihydrochloride reagents. Blank

tubes were processed as samples without NADH addition.

RESULTS

Exposure of maize seedling to 10μM Cd for 48 h resulted in almost 50% reduction of root growth. In contrast, Cd-treated plants supplemented with Mn showed lower Cd-induced root growth inhibition, magnitude of which was Mn-dose dependent (Figure 1). Application of 200μM Mn to root solution completely alleviated the toxicity of 10μM Cd on the root growth. The addition of 10, 100 or 200μM Mn alone, without Cd had no significant effect on root growth (data not shown).

The net NO_3^- uptake, both at low and high affinity uptake conditions was also reduced by 12 h pre-treatment with 10μM Cd. Magnitude of inhibition was similar for both systems and represented 26.3% for HATS and 28.9% for LATS. Pre-treatment with 100μM Mn was found to be slightly inhibitory for high affinity NO_3^- uptake (18.4% inhibition), but not for low affinity one (Figure 2). Furthermore, Mn slightly, but not significantly (P = 0.14) alleviated Cd-induced inhibition of NO_3^- uptake by LATS, but enhanced inhibition of NO_3^- uptake by HATS. Accumulation of NO_3^- in root tip tissues declined not only in the presence of Cd, but slightly also after Mn treatment, whereas Mn in combination with Cd did not show any improvement on nitrate accumulation (Figure 3).

Pre-treatment of maize roots with Cd showed a slight increase of the rest membrane potential value (Em) in contrary to Mn, pre-treatment with which caused a slight decrease of membrane potential (measured in the 1mM $CaSO_4$ solution, without Cd or Mn). After pre-treatment with both ions, the antagonistic effect of Mn to Cd on the membrane potential was observed. This effect was concentration dependent, and more-less equilibrated at 10:1 (Mn:Cd) stoichiometry (Table 1). Replacement of 1mM $CaSO_4$ by 1mM $Ca(NO_3)_2$ resulted in gradual hyperpolarization of membrane potential (Δ = 33.2 mV) (Figure 4). The initial electrical response to 2mM nitrate was significantly lower in plants pre-treated with Cd, pre-treatment with 100 and 200μM Mn alleviated the inhibitory effect of Cd.

The cadmium content in roots treated with Mn + Cd mixture was nearly the same as in the

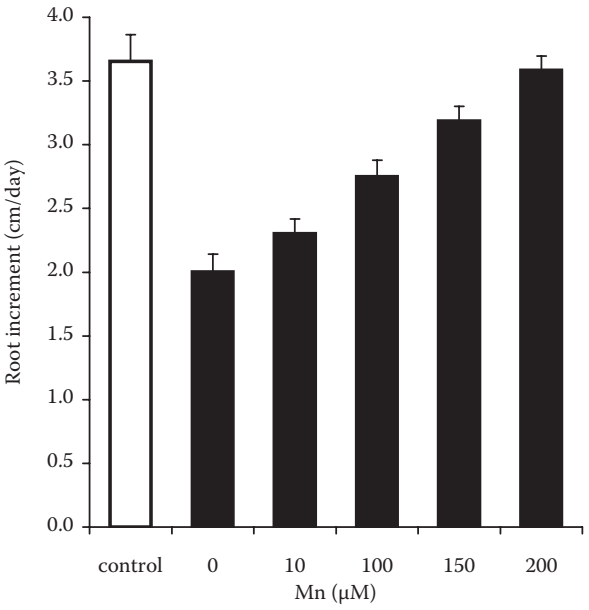

Figure 1. Effect of Cd and Mn ions on root growth of 3-day-old maize seedlings; root solution contained 0.5μM $CaSO_4$ (control – open column) supplemented with 10μM $CdCl_2$ and different concentrations of $MnCl_2$ (0–200μM – filled columns); the root growth increments are expressed in cm per day; results are the means (± *SE*) of 4 experiments (n = 20)

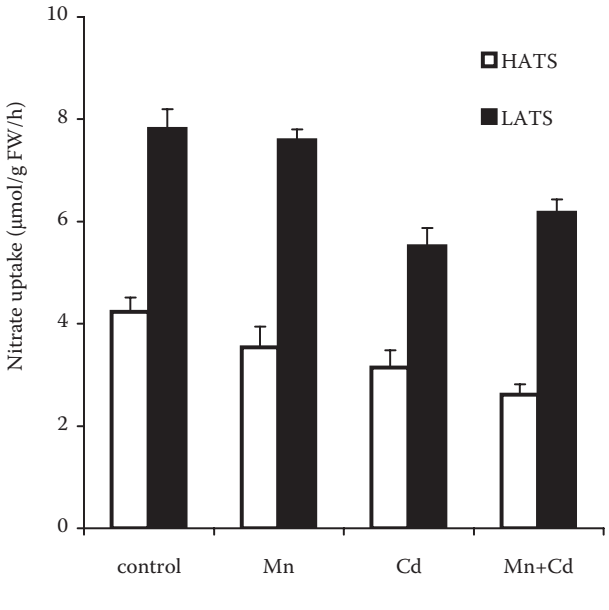

Figure 2. Nitrate uptake by roots of 14-day-old maize plants; twelve hours before the uptake measurement plants were transferred to solution containing 0.5mM $CaSO_4$, 0.2mM KNO_3, 10μM Cd and/or 100μM Mn; the uptake solution was Cd and Mn-free and contained 0.5mM $CaSO_4$ and 0.2mM KNO_3 for HATS or 2mM KNO_3 for LATS; results are the means (± *SE*) of 4 experiments (n = 3)

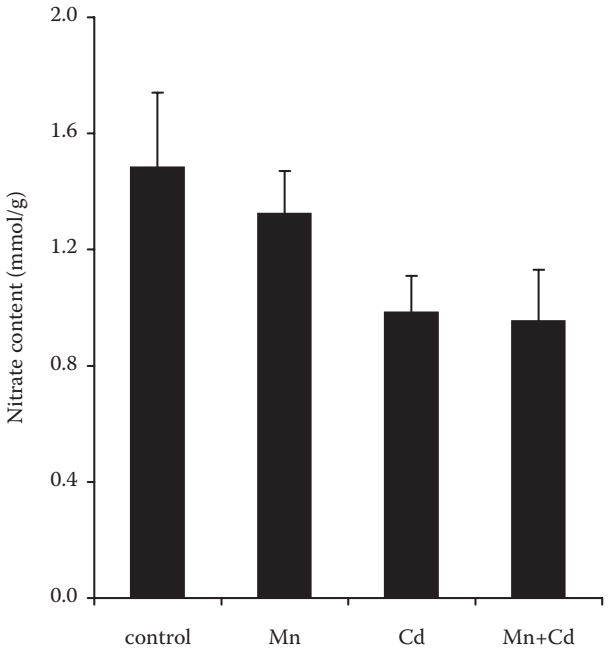

Figure 3. Nitrate content in 4 cm long apical segments of maize roots after 12 h pre-treatment with 10μM Cd (Cd), with 100μM Mn (Mn) or with a mixture of 10μM Cd + 100μM Mn (Mn + Cd); results are the means (± *SE*) of 4 experiments (n = 3)

plants treated with Cd alone, despite the 10 times higher concentration of Mn. On the other hand, the relatively low cadmium concentration in Mn + Cd media, led to the much stronger reduction (12–36%) of the manganese level in maize root tissue in comparison with the plants treated with Mn alone (Table 2).

Nitrate reductase activity (NRA) significantly decreased in roots treated by 10μM Cd. The extent of inhibition reached 28% in case of the total nitrate reductase activity (NRA_{tot}) and 39% in case of the actual nitrate reductase activity (NRA_{act}) (Figure 5). The same effect was observed in roots pre-treated with 100μM Mn. The extent of inhibition was very similar and reached 20 and 30% of both NRA_{tot} and NRA_{act}, respectively. The common effect of Mn + Cd was slightly synergic, resulting in 54% inhibition of NRA_{act}, and only 41% activity state of NRA (NRA_{act}/NRA_{tot}) in comparison to 63% activity state in control. The total protein content in root tissue remained unchanged during the NRA experiments.

DISCUSSION

Our experiments significantly confirmed that sufficient amount of Mn may fully ameliorate the Cd-induced root growth inhibition in maize seedlings. At the 1:1 stochiometry, which was in our conditions 10μM Mn and 10μM Cd, only weak alleviation effect of Mn was observed. The magnitude of Cd-toxicity alleviation increased with elevated Mn concentration and in the presence of 200μM Mn, when the Mn/Cd ratio reached 20:1, the root growth was approximately the same as in control plants. Such high Mn/Cd ratio could be usual in natural conditions, because natural Mn content in soils can reach mg/g range, even if the Mn availability for plants is partially reduced by Cd enrichment of the soil (Ramachandran and Souza 2002).

In spite of the fact that the total amelioration of Cd-induced root growth inhibition was observed after application of 200μM Mn concentration, further experiments (nitrate uptake and assimilation) were performed only with 100μM Mn. The main reason was that 200μM Mn alone negatively affected nitrate uptake and nitrate assimilation. Even a reduced concentration of Mn to 100μM

Table 1. Resting potential (Em) and hyperpolarization of membrane potential (Δ) during NO_3^- uptake in intact roots of maize seedlings pre-treated for 12 h with different concentrations of Cd and Mn

Treatment	Em (± *SE*)	Δ (± *SE*)
Control	–131.8 (± 1.0) c	–33.2 (± 1.2) c
100μM Mn	–126.9 (± 0.8) b	–29.1 (± 0.8) b
10μM Cd	–141.8 (± 1.0) e	–20.0 (± 0.5) a
10μM Cd + 10μM Mn	–142.6 (± 1.2) e	–18.9 (± 1.2) a
10μM Cd + 100μM Mn	–136.9 (± 1.2) d	–26.8 (± 1.2) b
10μM Cd + 200μM Mn	–122.9 (± 0.8) a	–26.1 (± 1.2) b

Significancy ($P_{0.05}$) is marked by letters

Table 2. Content of Mn and Cd in 4 cm root tips of maize seedlings in two separate experiments

Treatment	Cd content (μg/g DW)		Mn content (μg/g DW)	
	exp. 1	exp. 2	exp. 1	exp. 2
Control	4.2	7.6	4.4	4.2
Mn	n.d.	n.d.	411	419
Cd	370	323	n.d.	n.d.
Mn + Cd	312	307	263	326
% Mn + Cd/Cd	84.3%	95.0%	–	–
% Mn + Cd/Mn	–	–	64.0%	77.8%

n.d. = non detected; % Mn + Cd/Cd = the percentage of Cd content in roots treated with Mn + Cd to the Cd content in roots treated with Cd alone; % Mn + Cd/Mn = the percentage of Mn content in roots treated with Mn + Cd to the Mn content in roots treated with Mn alone

caused a significant inhibition of nitrate reductase activity.

Nitrate uptake in higher plants is mediated by two kinetically distinct systems. High-affinity transport system (HATS) works at low external NO_3^- concentrations, and low-affinity transport system (LATS), which is involved in nitrate uptake when external concentration of nitrate is higher than 1mM. Therefore, the uptake measurements at 0.2mM external NO_3^- represent the high affinity uptake conditions, and 2mM NO_3^- the low affinity uptake conditions. Both systems, HATS as well as LATS were significantly affected by Cd-treatment. In our experiments with maize, 12 h pre-treatment with 10μM Cd resulted in 28.5% reduction of nitrate uptake by LATS and 26.3% reduction by HATS. On the other hand, pre-treatment with both ions (Cd + Mn in ratio 1:10) almost doubled the inhibitory effect on HATS (39.4%) while partially but not significantly alleviated Cd-induced inhibition of LATS (21.7%). A similar reduction of nitrate uptake was demonstrated for wheat (Stolt and

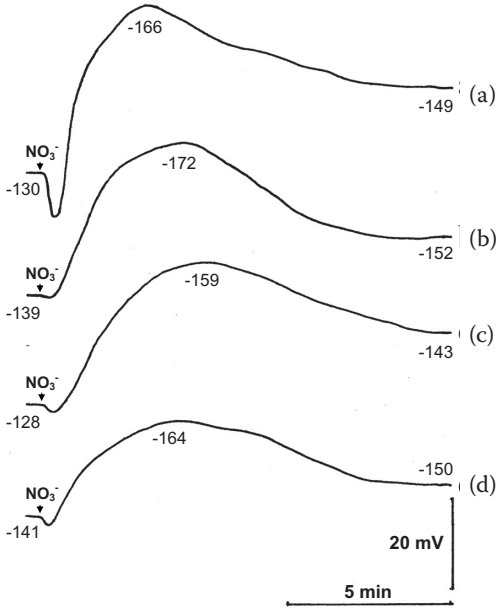

Figure 4. Representative changes of membrane potential of maize outer cortical root cells treated with 1mM $CaSO_4$ (a); 10μM Cd + 100μM Mn (b); 100μM Mn (c) or 10μM Cd (d); arrows indicate addition of 2mM $Ca(NO_3)_2$

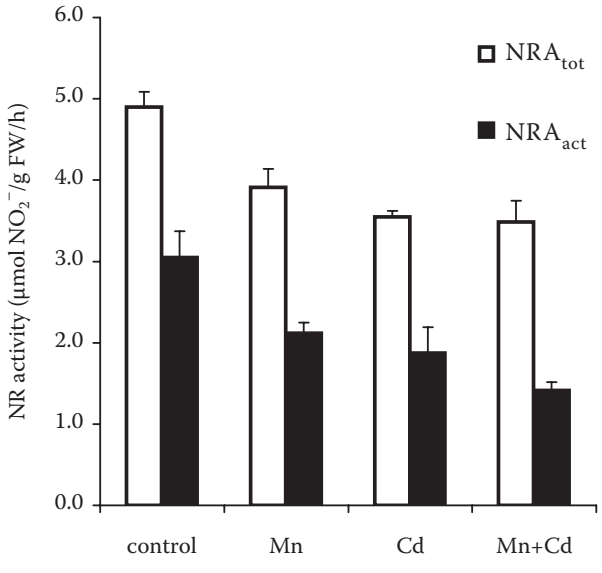

Figure 5. Total (NRA_{tot}) and actual activity (NRA_{act}) of nitrate reductase measured *in vitro* in 4 cm apical segments of maize roots pre-treated with 10μM Cd, 100μM Mn, or 10μM Cd + 100μM Mn; results are the means of 4 experiments (± *SE*, *n* = 3)

Oscarson 2002) and pea (Hernández et al. 1997). Boussama et al. (1999) firstly observed depression of the NO_3^- uptake after 12 h Cd pre-treatment during the NO_3^- induction period in barley, however, in this case 1mM NO_3^- was used for uptake measurements, which makes impossible to assign it to particular transport systems. This seems to be important for the effect of 100μM Mn, which was found to be slightly toxic for the HATS (18.2% reduction), but not for the LATS. Furthermore, a very slight alleviating effect of Mn was observed for LATS. The observed uptake at 2mM NO_3^- could be in fact a superposition of uptake provided by the LATS and HATS, and therefore alleviation effect of Mn for LATS could be partially masked by its slight toxicity for HATS.

Analysis of Mn and Cd content only partially confirmed the "competition theory" between Cd and Mn uptake (Rogers et al. 2000, Thomine et al. 2003, Cohen et al. 2004). In our experiments, plants treated for 12 h with solution contained 100μM Mn + 10μM Cd had lower Cd and Mn content than plants treated with Mn, or Cd alone as was reported by other authors (Jarvis et al. 1976, Baszyński et al. 1980, Wu et al. 2003). On the other hand, Mn-induced decrease of Cd content very low, and cannot explain the observed effects of Cd and Mn on nitrate uptake and assimilation.

To confirm the partially positive effect of Mn on Cd induced inhibition of LATS simultaneous electrophysiological measurements were performed on intact maize roots. Our electrophysiological measurements agree well with data presented by McClure et al. (1990) and Thibaud and Grignon (1981) working with intact maize roots. According to these authors, 2mM nitrate hyperpolarizes membrane potential to a value more negative than the original resting potential. Our results describe similar hyperpolarization and support a model for nitrate-dependent membrane potential hyperpolarization and nitrate uptake in co-transport with H^+. The magnitude of hyperpolarization was smaller in roots grown in presence of cadmium, and this effect was significantly alleviated in the presence of Mn, but only when Mn concentration was at least 10 times higher than Cd concentration. This observation is in good agreement with the results obtained by measurement of NO_3^- uptake upon depletion from the solution in low-affinity uptake conditions and supports the idea, that presence of Mn could decrease the toxic effect of Cd on LATS in root tissue.

As we mentioned earlier both Cd (10μM) and Mn (100μM) negatively affected NR activity. Reduction of NR activity was reported also in plants grown under Cd-stress (Hernández et al. 1997, Ouariti et al. 1997, Boussama et al. 1999). Our experiments showed that Cd significantly inhibits total (NRA_{tot}) as well as actual (NRA_{act}) NRA and this inhibition represents 28.3 and 38.0%, respectively. NR assayed in the presence of EDTA (NRA_{tot}) is proportional to the steady-state protein concentration. The assay in the presence of Mg^{2+} (NRA_{act}) gives an estimation of the in situ NR activity (Kaiser and Huber 1997). The presence of 100μM Mn alone decreased both NRA_{tot} and NRA_{act} in similar pattern, but magnitude of reduction was lower than in plants treated with Cd. The effect of both ions was synergic, when added together, to the NRA_{act} but not to the NRA_{tot}. It can be supposed, that both ions partially downregulated the NR on the protein synthesis level, but more strongly interfered with the posttranslational regulation level. The down regulation of NRA_{tot} could be at least partially explained by lower induction caused by lower root NO_3^- concentration after Cd treatment. On the other hand Mn, that had no significant effect on tissue NO_3^-, also decreased the NRA_{tot}.

Based on our results, we can conclude that an addition of excess amount of Mn to the solution containing phytotoxic concentrations of Cd can improve growth parameters of maize plants. The mechanisms involved in this improvement as well as in the alleviation of toxic effect of Cd on plants are still unclear, and are probably not connected to nitrate metabolism. Slight alleviation of Cd-induced inhibition of LATS by Mn can not overbalance severe toxicity of Cd on total nitrate uptake in short term experiments, but could be beneficial for long term plant growth and development. Therefore future experiments focused on long term effects of Cd and Mn ions on plant metabolisms may bring positive explanation of this problem.

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