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Interaction and accumulation of manganese and cadmium in the manganese accumulator *Lupinus albus*

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Summary

The effects of the interaction between Mn and Cd on the growth of the white lupin (*Lupinus albus*), its uptake of these metals, their accumulation, and on heavy metal stress indicators were studied under glasshouse conditions. Plants were grown with and without Mn and/or Cd for four weeks. The absence of Mn and Cd led to lipid peroxidation induced a loss of flavonoids and anthocyanins in the roots, reduced the size of the plant canopy, and led to the appearance of proteoid roots. Sensitivity to Cd in white lupin was enhanced by a low Mn supply, despite a lower Cd uptake and accumulation (leaf Mn:Cd concentration ratio <3), as shown by increased lipid peroxidation in the leaves and by the strong inhibition of growth. However, when the Mn supply was adequate, the plants showed few symptoms of Cd toxicity, even though Cd uptake and accumulation increased. A Mn:Cd ratio of up to 20 was enough to minimise Cd stress in the leaf, reflecting the plants' relative tolerance to Cd under such conditions. Irrespective of the Mn supply, the increase in antioxidant compounds observed in the roots of Cd-treated plants might act as a protective mechanism by minimising the oxidative stress caused by Cd exposure. In summary, high leaf Mn concentrations seem to render white lupins more tolerant to Cd stress.

KEYWORDS: Antioxidant compounds; Cadmium; *Lupinus albus* L.; Manganese; Stress indicators

Introduction

Manganese (Mn) is an essential trace element for plant growth; it plays an important role in enzyme activation, biological redox processes, in the splitting of water, and in the detoxification of oxygen free radicals (Marschner, 1995). Cadmium (Cd) is a dangerous heavy metal easily taken up by plants, and even at low levels usually causes injury; see Sanità di Toppi and Gabrielli (1999) for a review on Cd phytotoxicity. Many plant processes are strongly affected by Cd, including transport across membranes and photosynthesis, and it is associated with disturbances in the uptake and distribution of plant nutrients. Antagonism between Cd and Mn has been widely reported in plants stressed by Cd. Reductions in Mn uptake and accumulation in the shoots and roots have been reported in different plants e.g., soybean (Cataldo et al., 1983), lettuce (Thys et al., 1991), *Betula pendula* L. (Gussarsson, 1994), durum wheat (Jalil et al., 1994), pea (Hernández et al., 1998), cabbage, maize, white clover (Yang et al., 1998), and barley (Wu et al., 2003), when grown in Cd-polluted media. A low Cd concentration is, however, reported to increase Mn accumulation in the shoots, although not in the roots, of pea *(*Hernández et al., 1996) or lettuce (Ramos et al., 2002). In addition, Mn is thought to reduce Cd uptake in ryegrass (Jarvis et al., 1976) and soybean (Cataldo et al., 1983). Increasing Mn supplies to tomato (Baszynski et al., 1980) and maize (Pal'ove-Balang et al., 2006) is reported to be associated with parallel reductions in Cd uptake.

Manganese is often accumulated in the leaves of plants growing on acidic soils, with some plants accumulating Al as well (Reay and Waugh, 1981). Plants grown in neutral soils rarely accumulate Mn, except under anoxic conditions; in the white lupin (*Lupinus albus* L.), however, this is not the case. Manganese accumulation is a feature of *L. albus* leaves (up to 1 g Mn kg^{-1} dry weight), but this is not seen in *Lupinus angustifolius* L. or *Lupinus luteus* L. (Reay and Waugh, 1981; Reuter and Robinson, 1997). White lupin leaf Mn concentrations have been reported reduced in Cd-treated plants, although still higher than those thought associated with Mn deficiency, which might contribute to the mitigation of the injurious effect of Cd (Zornoza et al., 2002). Similarly, Ramos et al. (2002) reported strong Mn accumulation in the chloroplasts of leaves of Cd-treated lettuce showing no visual symptoms of Cd toxicity. Baszynski et al. (1980) even observed a partial restoration of Cd-induced chloroplast structural damage when tomato plants were transferred to a medium with excess Mn.

Despite the undertaking of numerous studies on the interaction of Cd and Mn in different plants, none has investigated this interaction in Mn starved plants. How Mn and Cd stress responses

are linked in white lupin, a Mn accumulator species, remains unclear. The present work tried to determine whether the Mn concentration of the leaves renders white lupins more or less tolerant to Cd stress. Differences in growth, Mn and Cd net uptake and accumulation were assessed in white lupin plants grown with and without Mn and/or Cd under controlled conditions. Malondialdehyde production and the concentrations of chlorophylls, total thiols, phenolic compounds, flavonoids and anthocyanins were used as indicators of Mn and Cd stress.

Materials and methods

Plant growth, Mn and Cd treatments

White lupin (*Lupinus albus* L. cv. Marta) seeds were surface-sterilised in 10% v/v sodium hypochlorite for 15 min, rinsed thoroughly with deionised water and germinated on water-moistened filter paper in the dark at 28°C for 3 days. The seedlings obtained were placed in plastic containers (8 L) with continuously aerated nutrient solution: 1.5 mM $Ca(NO_3)_2$, 4.0 mM KNO_3, 1.5 mM KH_2PO_4, 1.0 mM $MgSO_4$, 36 μM Fe-EDDHA, 33 μM $MnSO_4 \cdot H_2O$, 1.6 μM $ZnSO_4 \cdot 7H_2O$, 1.6 μM $CuSO_4 \cdot 5H_2O$, 46 μM H_3BO_3, 0.1 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (pH 5.5 - 6.0). All plants were grown in a glasshouse under the following environmental conditions: night/day temperature 12-28°C, a relative humidity of 50-80% and a photon flux density of 500 μmol m^{-2} s^{-1}. Ten days after sowing, four replicates of two Mn (0 and 33 μM $MnSO_4 \cdot H_2O$) and two Cd (0 and 18 μM $CdSO_4$) treatments were established using a randomised factorial design (Table 1). Deionised water was used for preparing all nutrient solutions and was added to replace transpiration losses every two days. The entire nutrient solutions were changed weekly and sampled. Plants were harvested at 0, 7, 14, 21 and 28 days after the initiation of treatments. The roots, stems and leaves of each plant were separated and their fresh weights (FW) recorded. They were then washed thoroughly with tap water three times, and again with deionised water three times. One thousand milligrams FW of plant material were frozen in liquid N_2 and stored at -20 ºC. The dry weight (DW) of the remaining plant matter was determined by oven-drying at 80ºC until a constant weight was achieved.

Element analyses of nutrient solutions and plant organs

The Mn and Cd concentrations of nutrient solution samples were analysed, without any further preparation of the latter, by atomic absorption spectrophotometry (Perkin-Elmer Analyst 800). The uptakes of these elements were calculated by their disappearance from the nutrient solution after

replacing water lost by transpiration. The concentration of these metals in plant organs (200 mg DW leaves, stems and roots) was determined by digesting samples with a mixture of HNO_3: H_2O_2: H_2O (3:2:10, v:v:v) for 30 min at 125ºC under a pressure of 1.5 kPa (Lozano-Rodríguez et al., 1995).

Stress indicators

Frozen samples were homogenised to a fine powder in liquid N_2 using an ice-cooled pestle and mortar. This material was then used to determine chlorophylls, lipid peroxides and total thiols (-SH). Leaf chlorophyll was extracted with 80% (v/v) acetone. The absorbance of the acetone extracts at 645 and 663 nm was determined and the chlorophyll *a* and *b* contents calculated (Wellburn, 1994). Malondialdehyde (MDA) and total thiols (-SH) were assayed as reported earlier (Esteban et al., 2008). Total phenolic compounds (PheC), flavonoids and anthocyanins were extracted from 200 mg oven-dried ground root samples with 10 mL of acidified methanol (0.1% HCl) and autoextracting at room temperature for 24 h. The acidified methanol was then replaced and the same procedure followed again. After centrifugation, the supernatant was adjusted to 25 mL with acidified methanol. The total phenolic content was determined using Folin-Ciocalteu reagent (Singleton and Rossi, 1965). To each tube, 0.25 mL of the extract was added followed by 3.75 mL of distilled water and 0.25 mL of the above reagent. After 3 min, 2 mL of 20% sodium carbonate were added. The tubes were capped, mixed thoroughly and heated at 40 ºC for 40 min. Blue coloration was read at 685 nm against a black standard. The results were expressed as mg of gallic acid g^{-1} DW of roots. Flavonoid concentrations were expressed as absorbance (A_b) at 300 nm g^{-1} DW, and anthocyanins calculated as A_b at 530 nm - 1/3 A_b at 657 nm g^{-1} DW (Lindoo and Caldwell, 1978).

Statistical analyses

The data presented are the means ± standard errors (S.E) of four replicates. To ensure that the assumptions for statistical analysis were fulfilled, the equality of variances and the normality of the data were tested. Differences between means for each variable were tested for significance by one- or two-way analysis of variance (ANOVA) as appropriate. Means were compared using the Duncan multiple range test ($P<0.05$). Significant differences among the individual treatments are expressed by different letters (a, b, c, d). Results of two-way ANOVA are expressed as NS (not significant) *$P<0.05$, **$P<0.01$ and ***$P<0.001$. All calculations were performed using SPSS v. 17.0 software.

Results

Visible effects and plant growth

After 28 days of growth, plants grown under the 0Mn+0Cd treatment had proteoid roots and showed slight inter-vein chlorosis in their young leaves. Chlorosis appeared both in the young and old leaves of plants in the 0Mn+18Cd treatment, accompanied by necrosis in the young leaves. Proteoid roots did not appear under the latter conditions, although those roots were a brownish colour. Plants in the 33Mn+18Cd treatment showed slight chlorosis in the young leaves only; the roots of these plants appeared similar to those of plants grown under the control (33Mn+0Cd) conditions. Moreover, all plants grown with Cd, with or without Mn, produced a small canopy. Table 2 summarises the root and shoot lengths and DWs of plants grown under the different Mn and Cd treatments after 28 days. Compared to the control plants (33Mn+0Cd), those of the 0Mn+18Cd treatment showed significant reductions in shoot (44%) and root (39%) length. In the 0Mn+0Cd and 33Mn+18Cd treatments, root length was not modified, nor was the shoot length of the 0Mn+0Cd plants. However, a significant 17% reduction in shoot length was recorded in the 33Mn+18Cd treatment. The DW of the shoots (leaves plus stems) of plants Cd-treated either with or without Mn (33Mn+18Cd and 0Mn+18Cd treatments) decreased significantly compared to the control (33Mn+0Cd). The absence of Mn and Cd (0Mn+0Cd treatment) also significantly reduced the DW of the leaves and stems (1.2-fold) compared to the control. Only the roots of the 0Mn+18Cd plants had lower DWs than the control (63%), while the 0Mn+0Cd and 33Mn+18Cd treatments had no significant effect on root DW (Table 2).

Two-way ANOVA of the organ length results showed significant differences between Mn and Cd treatments. The interaction *Mn x Cd* had a highly significant effect on shoot and root length. Two-way ANOVA of the DW results revealed highly significant differences between the Cd treatments and also between the Mn treatments (except with respect to leaf DW). The interaction *Mn x Cd* had a significant effect on root DW.

Net uptake and concentration of Mn and Cd

Figure 1 shows the cumulative net uptake of Mn and Cd by the roots of plants grown under the different Cd and Mn treatment conditions. The presence of Cd plus an adequate Mn level (33Mn+18Cd treatment) was associated with a strong reduction in net Mn uptake (Figure 1A)

,approximately 1.7 times lower than seen in the control treatment (33Mn+0Cd). Until 7 days of growth, the 33Mn+18Cd and 0Mn+18Cd plants took up similar amounts of Cd; thereafter Mn starvation reduced Cd net uptake significantly, becoming 2.1 times lower at 14 days, and 2.8 times lower at 21 and 28 days of growth than the Cd uptake shown by plants grown in the presence of Mn (Figure 1B). Moreover, the net Cd uptake of the 0Mn+18Cd plants remained fairly constant over the experiment. The Mn concentrations in the nutrient solutions for the 0Mn+0Cd and 0Mn+18Cd treatments, and the Cd concentrations in the 0Mn+0Cd and 33Mn+0Cd plants, were below detection limits. Plants grown under the 33Mn+0Cd treatment took up 63% of the total Mn supplied by day 28 of growth, whereas those of the 33Mn+18Cd treatment only took up 28%. The 33Mn+18Cd plants took up 45% of the total Cd supplied, while those of the 0Mn+18Cd only took up 16%.

Table 3 shows the concentrations of Mn and Cd in leaves, stems and roots of 28 day-old plants grown under the different Mn and Cd conditions. Compared to the control treatment (33Mn+0Cd), the concentration of Mn in the organs of the 0Mn+0Cd and 0Mn+18Cd plants was clearly reduced (>90%). Compared to the control treatment (33Mn+0Cd), the concentration of Mn decreased in the 33Mn+18Cd treatment, with reductions at the end of the experiment reaching 39%, 44%, and 61% in the leaves, stems and roots respectively. The leaf, stem and root Cd concentrations of the 0Mn+18Cd plants were significantly lower than those of the 33Mn+18Cd plants, with reductions of 66%, 31% and 32% respectively. Irrespective of the Mn supply, plants grown without Cd showed negligible concentrations of Cd (mg kg^{-1} DW) : <1.0 in leaves, <2.1 in stems and <2.8 in roots. Two-way ANOVA showed the Mn dose, Cd dose and the interaction *Mn x Cd* dose to significantly affect plant organ Mn and Cd concentrations (Table 3).

Chlorophyll concentrations and stress indicators

Table 4 shows the leaf concentrations of the studied chlorophylls and those of root total PheC, flavonoids and anthocyanins in 28 day-old plants grown under the different treatments. Compared to the control (33Mn+0Cd), chlorophyll *a* was significantly reduced in the leaves of the 33Mn+18Cd (19%) and 0Mn+18Cd (51%) plants. Chlorophyll *b* only suffered a significant reduction in the leaves of 0Mn+18Cd plants (42%). In contrast, Cd-untreated plants, either with or without an adequate Mn supply, gave similar chlorophyll concentrations. Compared to the control plants, the concentration of total PheC increased significantly in the roots of the 33Mn+18Cd and 0Mn+18Cd plants by about 16% (no significant difference between them). No difference was seen in the concentration of total PheC

between control (33Mn+0Cd) and 0Mn+0Cd treatments. Compared to control plants, total flavonoids increased (16%) and decreased (13%) significantly in the roots of the 0Mn+18Cd and 0Mn+0Cd plants respectively; no differences were seen between the control and 33Mn+18Cd treatments. Anthocyanins declined significantly in the roots of the 0Mn+0Cd plants (25%); the other treatments returned values similar to that of the control.

Leaf and root MDA and -SH levels of plants grown under the different Cd and Mn treatments are shown in Figures 2 and 3, respectively. Compared to the control plants, MDA increased significantly both in the leaves (44%) and roots (91%) of the 0Mn+18Cd plants and in the leaves (26%) and roots (35%) of the 0Mn+0Cd and 33Mn+18Cd treatments respectively. No significant differences were seen in leaf and root total -SH contents between the control (33Mn+0Cd) and 0Mn+0Cd treatments, whereas they increased 6 and 4.9 times in the roots of the 33Mn+18Cd and 0Mn+18Cd plants respectively. However, in the leaves of Cd-treated plants, grown with or without Mn, the –SH content decreased.

Two-way ANOVA showed significant differences between Cd treatments with respect to chlorophyll and stress indicators (except for leaf MDA). Mn supply was found to have a significant effect on chlorophyll *a*, MDA and -SH in the leaf, as well as on root anthocyanins. The interaction *Mn x Cd* had a significant effect on the leaf chlorophyll *a* and on root MDA, total -SH and flavonoids.

Discussion

Growth inhibition is frequently observed in higher plants exposed to Cd, although the severity of Cd stress symptoms depend largely on the capacity of plants to tolerate this heavy metal. Visible symptoms of Cd injury in plants often include leaf chlorosis and necrosis, the discoloration of leaf blades, browning of the root tips, and finally death (Sanità di Toppi and Gabbrielli, 1999). Root browning is reported to be due to the enhanced suberization or lignification of the root tips, and a consequent loss in nutrient uptake capacity (Schützendübel et al., 2001). In this study, the supply of Cd to Mn-starved plants had negative effects on plant growth, accompanied by strong reductions in leaf chlorophylls, with the roots turning a brownish colour. In contrast, Cd had no effect on root development when an adequate Mn supply was available, although slight symptoms of Cd toxicity were visible in the shoots of these plants (Tables 2 and 4). Previous investigations into Cd stress in white lupins grown with Cd doses of ≤45 μM found virtually no growth problems (Zornoza et al., 2002).

Since the roots are probably the first to suffer Cd injury, this non-inhibition of root growth might be a sign of relative Cd tolerance by this species when an adequate Mn supply is available.

The Mn requirements of the majority of crop plants are satisfied at tissue levels of around 20-40 mg Mn per kg^{-1} DW (Reisenauer, 1988). However, for white lupin leaves a range of 318-1300 mg kg^{-1} DW is considered adequate, whereas values of <55 mg kg^{-1} DW are deficient (Reuter and Robinson, 1997). In the present study, the Mn-starved plants (Table 3) had very low Mn concentrations (40-60 mg kg^{-1} DW) after 28 days without Mn, values below the critical level for white lupins (Reuter and Robinson, 1997). In response to Mn starvation, white lupins develop special root clusters; however, these did not appear when no Mn was provided in the presence of Cd. The formation of proteoid roots appears to be mainly induced by a shortage of P and, at least in some plant species, by Fe deficiency (Dinkelaker et al., 1995; Hagström et al., 2001). Earlier studies into Cd stress in white lupins grown with Cd doses of ≤45 μM found strong reductions of P and Fe plant accumulation, but proteoid roots did not appear in Cd stressed plants (Zornoza et al., 2002).

A specific negative Cd-Mn correlation has been widely reported in plants visually stressed by Cd (Hernández et al., 1998; Yang et al., 1998; Gussarsson, 2004). In the present study, the supply of Cd inhibited total Mn net uptake by some 45% in plants grown with Mn in the nutrient solution and reduced to almost half the Mn concentration of their organs compared to those not Cd-treated (Figure 1A; Table 3). Similarly, the absence of Mn in the nutrient solution of the Cd-treated plants reduced Cd net uptake and plant concentration to below those seen when an adequate Mn level plus Cd was made available (Figure 1B; Table 3). It has been reported that Cd and Mn share common transport systems in plants (Hart et al., 1998; Clemens et al., 2002). However, the effects of Mn starvation reducing Cd uptake and plant concentrations should be noted, indicating that this Mn-Cd interaction was not owed to true cation antagonism. Further, Cd, Cu, Fe, Zn and possibly Mn share a common transport site or process in Cd-treated soybean (Cataldo et al., 1983), tomato (Baszynski et al., 1980) and *Phytolacca americana* L. (a Mn-Cd hyperaccumulator) (Peng et al., 2008).

The accumulation of Cd stopped in the organs of plants grown under Mn starvation conditions; this was most remarkable in the leaves, in which the Cd concentration hardly changed over time (data not shown). This suggests that, despite the low net Cd uptake under Mn deficiency, the capacity of the root to retain Cd is reduced, and Cd is easily transported from roots to shoots, increasing the symptoms of Cd phytotoxicity in these plants (Tables 2 and 4). Nevertheless, under an adequate Mn

supply, Cd-treated white lupins showed a greater Cd net uptake and accumulation, although this proved not to be toxic; the plants therefore showed a certain Cd tolerance. Reducing the Mn supply to the plants promoted a reduction in Cd uptake, although the opposite has been reported by other authors (Baszynski et al., 1980; Pal'ove-Balang et al., 2006).

Plant cells activate different detoxification mechanisms to avoid Cd stress, such as binding the Cd by phytochelatins, accumulating it in cell organelles, immobilising it in cell walls, and the synthesis of stress proteins (Sanità di Toppi and Gabbrielli, 1999). Oxidative stress promoted by Cd exposure, possibly by the generation of free radicals and active oxygen species, might cause lipid peroxidation. MDA provides an index of lipid peroxidation and, therefore, of oxidative stress. Increases in MDA caused by Cd exposure have been widely observed (Sandalio et al., 2001; Schützendübel et al., 2001). In the present work, supplying Cd led to increased MDA root concentrations in both Mn-supply treatments, but only in the shoots of plants grown without Mn (Figure 2). This might indicate a higher peroxidation tolerance in white lupin roots grown with an adequate Mn supply than that shown by Mn-starved plants.

Thiol groups play an important role in the cytoplasmic detoxification defence mechanism against heavy metals, but they are also required to counteract the harmful effects of oxidative stress (Noctor and Foyer, 1998). Phenolic compounds are secondary metabolites that protect plant tissues from oxidative damage because of their antioxidant capacity; their accumulation in plants is stimulated by various biotic and abiotic stresses (Dixon and Paiva, 1995). A strong increase was seen in -SH (up to 4.9-fold) along with less strong increases in PheC in the roots of Cd-treated plants grown with or without Mn (Figure 3). The increase in these compounds could be a defence mechanism developed by the roots of the white lupin to minimize the oxidative damage caused by Cd exposure, reflected in enhanced MDA production (Figure 2). Previous reports have shown a close relationship between the accumulation of PheC and overall plant resistance to a number of heavy metals, e.g., Cu in alfalfa (Parry et al., 1994) and Cu (Jung et al., 2003) and Hg (Esteban et al., 2008) in the white lupin. In addition, the increase in flavonoids in the roots of plants grown without Mn but with Cd might indicate that defence mechanisms against Cd toxicity are intensified since Mn deficient plants showed Cd hypersensitivity.

Conclusions

The present results indicate that, despite lower Cd uptake and accumulation (leaf Mn:Cd concentration ratio <3), sensitivity to Cd stress in the white lupin was enhanced by a low Mn supply, as shown by the appearance of leaf and root oxidative stress that strongly inhibited growth. On the contrary, an adequate Mn supply led to high Cd and Mn accumulation (leaf Mn:Cd concentration ratio > 20), resulting in few symptoms of Cd toxicity in these plants. This implies a protective role of Mn in photosynthetic tissues. The associated increase in the antioxidant capacity of the root together with a high leaf Mn concentration might be responsible for the relative Cd tolerance observed in this Mn-accumulator legume. Future work should investigate the kinetic parameters of Cd uptake in the presence and absence of Mn in white lupins.

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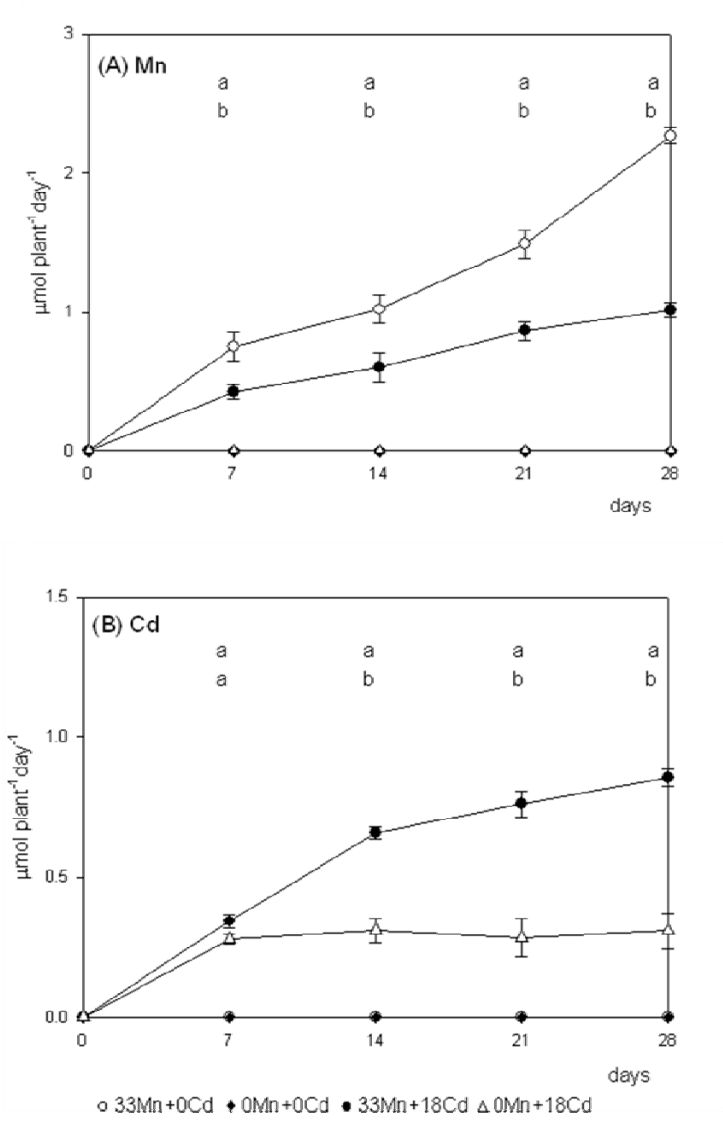
Legend of figures

Figure 1. Cumulative net uptake of Mn (A) and Cd (B). Each data point represents the mean (with S.E. bar) of four replicates. Where no bar is visible, the S.E. is smaller than the data point. Different letters above the bars indicate significant differences among Mn and Cd treatments ($P<0.05$).

Figure 2. Effect of Mn and Cd treatments on MDA concentrations (nmol g^{-1} FW) in 28 day-old plants (mean ± S.E.). Different letters above the bars indicate significant differences among Mn and Cd treatments ($P<0.05$).

Figure 3. Effect of Mn and Cd treatments on -SH concentrations (nmol SH g^{-1} FW) in 28 day-old plants (mean ± S.E.). Different letters above the bars indicate significant differences among Mn and Cd treatments ($P<0.05$).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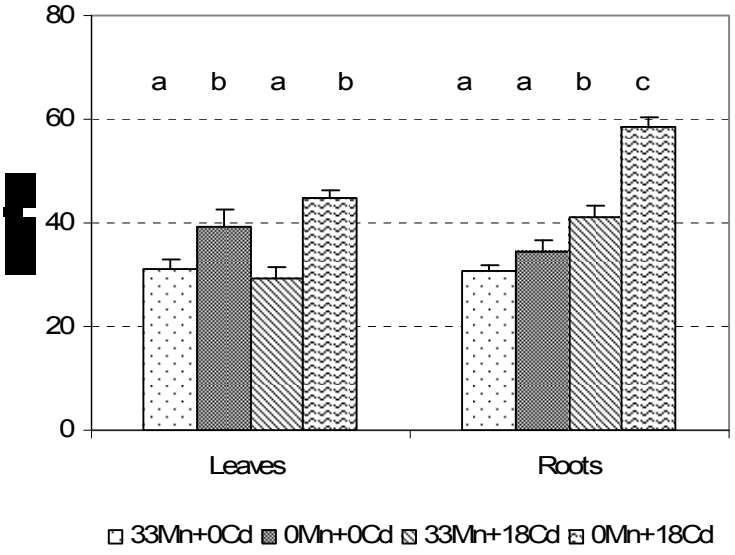
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33Mn+18Cd
0Mn+18Cd

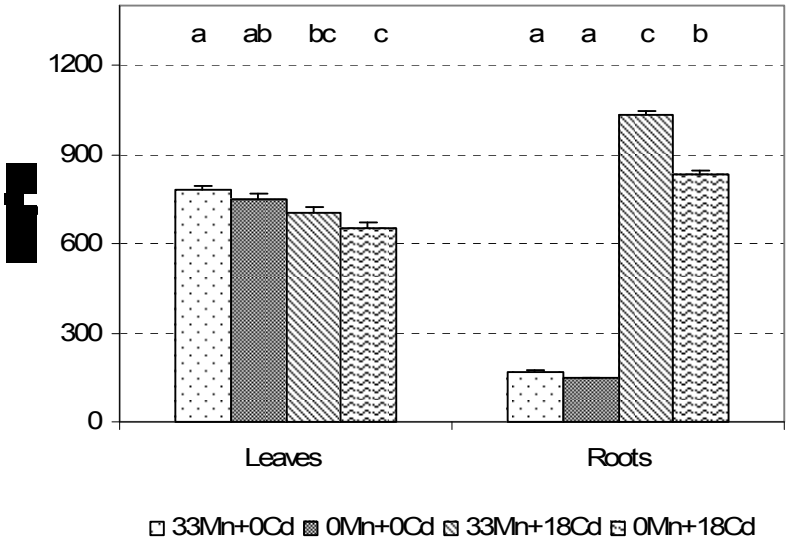
a ab bc c
a a c b
1200
900
600
300
0
Leaves
Roots
33Mn+0Cd
0Mn+0Cd
33Mn+18Cd
0Mn+18Cd

Table 1. Treatments undertaken.

Treatments	Abbreviation used	Mn and Cd doses
Adequate Mn (control)	33Mn+0Cd	33 µM Mn + 0 µM Cd
Without Mn	0Mn+0Cd	0 µM Mn + 0 µM Cd
Adequate Mn plus Cd	33Mn+18Cd	33 µM Mn + 18 µM Cd
Without Mn plus Cd	0Mn+18Cd	0 µM Mn + 18 µM Cd

Table 2. Effect of Mn and Cd treatments on organ length and dry weight of 28 day-old plants (± S.E.). Means in the same row followed by the same letter do not differ significantly according to the Duncan test (P< 0.05). Two-way ANOVA results: NS, not significant, *P<0.05, **P<0.01, ***P<0.001.

	Treatments				**Two-way ANOVA *F* results**		
Plant part	**33Mn+0Cd**	**0Mn+0Cd**	**33Mn+18Cd**	**0Mn+18Cd**			
	Length (cm plant^{-1})				Mn	Cd	Mn x Cd
Shoot	22.59 ± 0.37^{a}	21.97 ± 0.29^{a}	18.79 ± 0.18^{b}	12.59 ± 0.11^{c}	***	***	***
Root	48.00 ± 0.86^{a}	44.90 ± 0.60^{a}	45.80 ± 0.49^{a}	29.20 ± 0.35^{b}	***	***	***
	Dry weight (g plant^{-1})				Mn	Cd	Mn x Cd
Leaf	1.32 ± 0.06^{a}	1.08 ± 0.08^{b}	0.51 ± 0.04^{c}	0.50 ± 0.04^{c}	NS	***	NS
Stem	1.06 ± 0.08^{a}	0.84 ± 0.05^{b}	0.37 ± 0.02^{c}	0.30 ± 0.01^{c}	**	***	NS
Shoot	2.38 ± 0.15^{a}	1.92 ± 0.12^{b}	0.88 ± 0.08^{c}	0.80 ± 0.07^{c}	*	***	NS
Root	0.84 ± 0.05^{a}	0.81 ± 0.06^{a}	0.75 ± 0.03^{a}	0.31 ± 0.02^{b}	***	***	**

Table 3. Effect of Mn and Cd treatments on Mn and Cd concentrations (mg kg^{-1} DW) in 28-day-old plants (mean ± S.E.). Means in the same row followed by the same letter do not differ significantly according to the Duncan test (P<0.05). Two-way ANOVA results: NS, not significant, *P<0.05, **P<0.01, ***P<0.001.

		Treatments				**Two-way ANOVA *F* results**		
	Plant organ	**33Mn+0Cd**	**0Mn+0Cd**	**33Mn+18Cd**	**0Mn+18Cd**	Mn	Cd	Mn x Cd
Mn	Leaves	1389.6±12.5^{a}	59.2± 5.3^{c}	846.5±11.5^{b}	37.0± 1.2^{c}	***	***	***
	Stems	336.0± 8.3^{a}	16.0± 1.7^{c}	188.5± 6.9^{b}	5.2± 0.5^{d}	***	***	***
	Roots	242.9± 5.4^{a}	24.0± 0.6^{c}	95.2± 1.1^{b}	8.4± 0.2^{d}	***	***	***
Cd	Leaves	0.2± 0.1^{c}	0.8± 0.2^{c}	82.1± 1.2^{a}	27.6± 1.3^{b}	***	***	***
	Stems	0.5± 0.1^{c}	1.9± 0.2^{c}	161.1± 4.1^{a}	111.0± 3.5^{b}	***	***	***
	Roots	1.6± 0.1^{c}	2.5± 0.3^{c}	1693.5±15.3^{a}	1151.3± 9.1^{b}	***	***	***

Table 4. Effect of Mn and Cd treatments on leaf chlorophyll concentrations (mg g^{-1} FW) and total PheC (mg g^{-1} DW), flavonoids (A_b g^{-1} DW) and anthocyanins (A_b g^{-1} DW) in roots of 28 day-old plants (mean ± S.E.). Means in the same row followed by the same letter do not differ significantly according to the Duncan test (P<0.05). Two-way ANOVA results: NS, not significant; *P<0.05; **P<0.01; ***P<0.001.

	Treatments				**Two-way ANOVA**		
	33Mn+0Cd	**0Mn+0Cd**	**33Mn+18Cd**	**0Mn+18Cd**	***F* results**		
			Leaves		Mn	Cd	Mn x Cd
Chlorophyll *a*	0.72 ± 0.01[a]	0.65 ± 0.02[a]	0.58 ± 0.01[b]	0.35 ± 0.03[c]	***	***	**
Chlorophyll *b*	0.24 ± 0.01[a]	0.24 ± 0.01[a]	0.22 ± 0.01[a]	0.14 ± 0.01[b]	NS	**	NS
			Roots				
Total PheC	16.24 ± 0.48[a]	15.79 ± 0.25[a]	18.71 ± 0.39[b]	19.04 ± 0.59[b]	NS	**	NS
Flavonoids	43.31 ± 0.72[a]	37.64 ± 0.81[b]	44.75 ± 0.69[a]	50.46 ± 0.75[c]	NS	***	**
Anthocyanins	0.36 ± 0.05[a]	0.27 ± 0.02[b]	0.40 ± 0.02[a]	0.42 ± 0.02[a]	*	**	NS