Growth of spinach plants (*Spinacia oleracea* L.) exposed to excess zinc and manganese

Crecimiento de plantas de espinaca (*Spinacia oleracea* L.) expuestas a exceso de zinc y manganeso

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ABSTRACT

Increased anthropogenic inputs of heavy metals in agricultural soils lead to worrisome effects on the water, soil, and plants. A greenhouse study was conducted in Tunja, Colombia to determine the effects of excess Zn and Mn on leaf area, root length, dry matter production and partitioning, root to shoot ratio, specific leaf weight, water uptake, and agronomic water use efficiency in spinach seedlings (*Spinacia oleracea* L. hyb. Marimba). Seedlings were grown floating on a complete nutrient solution in 4 L glass containers. Concentrations of 40 mg L^{-1} Mn, 40 mg L^{-1} Zn, 80 mg L^{-1} Mn, 80 mg L^{-1} Zn and combinations thereof were added to the solution, with a control treatment receiving no excess Zn or Mn. Zn at 40 mg L^{-1} reduced leaf area by 78.82% in relation to the controls; 40 mg L^{-1} Mn increased leaf area by 35.23%. Plants exposed to 80 mg L^{-1} Zn with 80 mg L^{-1} Mn increased allocation of biomass to leaves by 45.05% as compared to the control plants. Addition of 80 mg L^{-1} Zn and 40 mg L^{-1} Mn + 80 mg L^{-1} Zn led to an increase of 9.24 and 29.75%, respectively, in dry matter allocation to stem + petioles. Roots were affected the most by excess Mn and Zn, alone and in combination. While addition of 40 or 80 mg L^{-1} of Mn reduced total root length by 45.06 and 81.64%, respectively; while Zn concentrations of 40 or 80 mg L^{-1} reduced total root length by 88.78 and 98.07%, respectively.

Key words: water uptake, dry matter partitioning, root length, heavy metals.

RESUMEN

El aporte antrópico de metales pesados en suelos agrícolas causa efectos preocupantes en agua, suelos y plantas. Se realizó un estudio en invernadero en Tunja, Colombia para determinar el efecto del exceso de Mn y Zn sobre el área foliar, la longitud total de la raíz, la producción y distribución de materia seca, la relación de raíz: vástago, el peso foliar específico, el consumo de agua y la eficiencia agronómica en el uso del agua, en plantas de espinaca (*Spinacia oleracea* L. hyb. Marimba). Las plantas se cultivaron flotando sobre 4 L de una solución nutritiva completa. A algunos contenedores se les adicionó 40 u 80 mg L^{-1} de Mn, 40 u 80 mg L^{-1} de Zn mg L^{-1}, o las combinaciones entre estas concentraciones, con un control sin exceso de Zn o Mn. A 40 mg L^{-1} Zn redujo 78,82% el área foliar en relación con los controles; con 40 mg L^{-1} Mn aumentó el área foliar 35,23%. Las plantas expuestas a 80 mg L^{-1} Zn + 80 mg L^{-1} Mn aumentaron 45.05% la asignación de biomasa a las hojas. La adición de 80 mg L^{-1} Zn y 40 mg L^{-1} Mn + 80 mg L^{-1} Zn condujo a un aumento 9,24 y 29,75%, respectivamente, de la asignación de materia seca a tallo + pecíolos. Las raíces fueron las más afectadas por el exceso de Mn y Zn, solos y combinados. La adición de 40 u 80 mg L^{-1} de Mn redujo la longitud total de las raíces 45,06 y 81,64%, respectivamente; Zn a 40 u 80 mg L^{-1} la disminuyó 88,78 y 98,07%.

Palabras clave: toma de agua, distribución de la masa seca, longitud radical, metales pesados.

Introduction

Heavy metals are ubiquitous in the environment as a result of both natural and anthropogenic activities; humans, plants, and animals are exposed to them through various pathways (Wilson and Pyatt, 2007). Wastewater irrigation, solid waste disposal, sludge applications, vehicular exhaust and industrial activities are major sources of soil contamination by heavy metals, and increased metal uptake by food crops grown on such contaminated soils is often observed (Singh *et al*., 2004; Chen *et al*., 2005). Excessive contents of metal ions in plants can cause various stress responses manifested outwardly with visible symptoms such as growth inhibition, root damage, chlorosis, larger stomata, and thinning of wax deposition. Rapid physiological and slow morphological processes cause changes in plant metabolism, directly or indirectly related to plant "management" of metal stress (Schützendübel and Polle, 2002; Rai *et al*., 2005; Sharma and Dietz, 2006; Casierra-Posada *et al*., 2010). Mn toxicity is more common than Mn

deficiency in some regions such as the humid tropics, where most soils are highly weathered and acidic (Hue *et al.*, 2001).

Municipal solid waste management in Bogota, Colombia is carried out as a public service that includes collection, transport, and disposal. The waste is disposed of in the sanitary landfill Doña Juana located south of the city. It receives an average 6,000 t d^{-1} of municipal solid waste; the Bogota Capital District is in charge of the management of the landfill. There is no formal recycling system for the city, a fact that has social, environmental, and economic consequences (Hincapié and Mantilla, 2007). Thus, the waste disposed of in the sanitary landfills becomes an environmental problem and contaminates soil and water with heavy metals, among other compounds.

In horticultural crops established in the Bogota Plateau, farmers irrigate with water from the Bogota river, through the "La Ramada" irrigation district , which covers 6400 ha of land. The use of this water has generated a contamination problem in these crops, causing quality deterioration of the produce, which is mainly consumed fresh. In a study carried out in plantations of lettuce, celery, cabbage, and broccoli in Soacha (Colombia) , heavy metals such as lead, cadmium, arsenic and mercury in water, soil, and edible plant parts were analyzed. Lettuce and celery plants were contaminated with Cd exceeding the limits of the European Union norms. In all evaluated vegetables, Pb contents exceeded the maximum allowed concentration in foods for breastfeeding infants and young children. Lettuce plants accumulated higher levels of heavy metals than the other three vegetable species (Miranda *et al.*, 2008).

Although in the vegetable-growing area of Soacha, Colombia it is not common practice to apply municipal waste compost to crops, much of the scientific work done on heavy metal toxicity in plants involves application of such compost. Toxic effects of zinc on plant growth have been reported for spinach (Pavlíková *et al.*, 2008). However, metal-enriched spinach plants have a detoxification mechanism and grow well on organic matter-enriched soil contaminated with toxic metals (Sinha *et al.*, 2007). Acid soils have a larger pool of available Mn than neutral or alkaline soils; the addition of municipal solid waste compost to an acidic sandy loam increased Mn in blueberry leaves (Warman *et al.*, 2004). Increased spinach uptake of Mn due to application of municipal solid waste compost has also been observed in calcareous soil (Maftoun *et al.*, 2004). Gallardo-Lara *et al.* (2006) reported an increase in lettuce Mn and reduced Mn in barley in calcareous soil amended with the same compost. Swiss chard and basil

Mn tissue concentrations decreased with municipal solid waste compost additions. That is to say, that the highest Mn uptake was recorded at the lowest application rate and the lowest uptake at the highest application rate (Zheljazkov and Warman, 2004b).

Hargreaves *et al.* (2008) have done a review of relevant agricultural studies as well as recommendations for improving municipal solid waste compost quality. Its safe use in agriculture can be ensured with source separation (or sorting of municipal solid waste to be composted) as well as the development and implementation of comprehensive industry standards. Despite this, the composts used in some studies have very high concentrations of Cu and Zn. Zinc uptake by potatoes, swiss chard, and basil grown in soil treated with municipal solid waste compost has been reported on (Sebastiao *et al.*, 2000; Zheljazkov and Warman, 2004a). However, despite increases in available soil Zn, the Zn concentration of tomato and squash fruit, as well as grapevines, did not increase with municipal solid waste compost additions (Ozores-Hampton and Hanlon, 1997; Pinamonti *et al.*, 1999).

The largest portion of Mn in soil treated with municipal solid waste compost was found to be bound in the iron-manganese fraction, which is unavailable to plants (Zheljazkov and Warman, 2004a). Interactions between Fe and Mn availability were also reported (Maftoun *et al.*, 2004). There is usually a decreased plant availability of Mn as a result of municipal solid waste compost addition because of the increase in soil pH associated with municipal solid waste compost application (Warman *et al.*, 2004).

Small increases of total soil Zn concentrations were observed where municipal solid waste compost was applied at a rate of 15 t ha^{-1} to an alkaline sandy soil, but no increases were seen when the compost was applied to an acidic sandy soil or a neutral sandy loam (Sebastiao *et al.*, 2000). In a pot experiment with *Beta vulgaris*, municipal solid waste compost increased total soil Zn concentration, but most of the Zn was bound in the iron–manganese fraction of the soil and plant uptake was reduced (Zheljazkov and Warman, 2004b). Furthermore, water soluble Zn has been observed to become immobilized in soil with the addition of municipal solid waste compost (Hernando *et al.*, 1989).

The present study was conducted to compare the effect of heavy metals (zinc and manganese and their interactions) on growth parameters of spinach plants grown in bottles

filled with a nutrient solution to which high contents of zinc and manganese were added.

Materials and methods

Plant growth conditions

The study was conducted in a greenhouse at the Universidad Pedagógica y Tecnológica de Colombia (UPTC) in Tunja, Colombia (5°33'16.25" N; 73°21'9.14" O) at 2,790 m a.s.l. Seeds of *Spinacia oleracea* L. hybrid Marimba (Seminis, Spain) were sown in a mixture 1:1 of soil and peat. Average growth conditions inside the greenhouse were: photosynthesis photon flux 782.2 µmol m^{-2} s^{-1}, 15.8°C temperature and 72% relative humidity. Four weeks after germination, 90 single seedlings (ten plants for each treatment) were transplanted to styrofoam cubes floating on a nutrient solution in 4 L glass containers. The solution's content in mg L^{-1} was: NO_3 40.3; NH_3 4.0; P 20.4; K 50.6; Ca 28.8; Mg 11.4; S 1.0; Fe 1.12; Mn 0.112; Cu 0.012; Zn 0.0264; B 0.106; Mo 0.0012 and Cu 0.00036. The average pH and EC values of the solution were 5.8 and 2.0 mmhos cm^{-1} respectly. The nutrient solution was prepared adding 1 cm^3 of Nutriponic® (Walco S.A.) per liter of water and was aerated with an aquarium air pump during the entire growth cycle of the plants. After transplant, when the seedlings reestablished growth, Zn and Mn from $ZnSO_47H_2O$ or $MnSO_4H_2O$ (Merck®), respectively, were added to the nutrient solution.

Growth measurements

Plants were harvested 50 d after transplant. The agronomic efficiency of water use (g L^{-1}) was calculated as the amount of water needed to produce one gram of dry matter. Water consumption (L) was taken as the amount of water added periodically to the bottles. Leaf area was measured using an LI-3000. A analyzer (LI-COR, Lincoln, NE, USA). For determination of total root length (m), roots were dipped in formaldehyde and blue methylene to preserve them and make viewing easier; roots were sectioned and formed into a line and measured as if they were a single long root. Then the plants were dried to determine total dry weight and dry weight of each plant organ (roots, petioles + stem and leaves), by drying them in an oven at 70°C for 48 h. The specific leaf weight (mg cm^{-2}) was obtained as a ratio between the total dry weight of the leaves and the leaf area of the same plant. Finally, the root to shoot ratio was calculated as the relation between the root dry weight and dry weight of the aerial organs (leaves, petioles and stems) of each plant.

Statistical analysis

All factors were evaluated in a completely randomized design with nine treatments: 0 Mn + 0 Zn (controls); 40 mg L^{-1} Mn + 0 mg L^{-1} Zn; 0 Mn + 40 mg L^{-1} Zn; 80 mg L^{-1} Mn + 0 Zn; 0 Mn + 80 mg L^{-1} Zn; 80 mg L^{-1} Mn + 40 mg L^{-1} Zn; 40 mg L^{-1} Mn + 80 mg L^{-1} Zn; 40 mg L^{-1} Mn + 40 mg L^{-1} Zn and 80 mg L^{-1} Mn + 80 mg L^{-1} Zn. For measurement of the growth parameters, all 10 seedlings of each treatment were used. Each seedling was treated as a repetition. All the data sets obtained from the experiment were subjected to two way analysis of variance (ANOVA) using SPSS 17.0.0, taking $P \leq 0.01$ as significant, followed by a post hoc Tukey test.

Results and discussion

Leaf area was affected by high contents of Mn and Zn in the nutrient solution. For this parameter, a significant difference ($P \leq 0.01$) was found. While Zn at a concentration of 40 mg L^{-1} reduced leaf area by 78.82% in relation to the controls, for the same concentration of Mn, leaf area increased 35.23% over the average value of the controls. In plants exposed to 80 mg L^{-1} of Mn, leaf area was reduced by 11.66% compared to the controls (though this did not comprise a significant difference), but with an addition of Zn 80 mg L^{-1}, the reduction was 94.52%. Although no significant difference was found between treatments combining both Zn and Mn, it was observed that the toxic effects induced by Zn on the development of leaf area were less severe with higher Mn concentration in the nutrient solution (Fig. 1).

The results presented in Fig. 1 can be explained by the antagonistic interaction between Zn and Mn. Some reviews cite ion antagonism in plants between Ca and Zn; Fe with Ca, Zn, Pb, Cd, Mn, or Cu; Mn with Zn or Ca; K with Na,
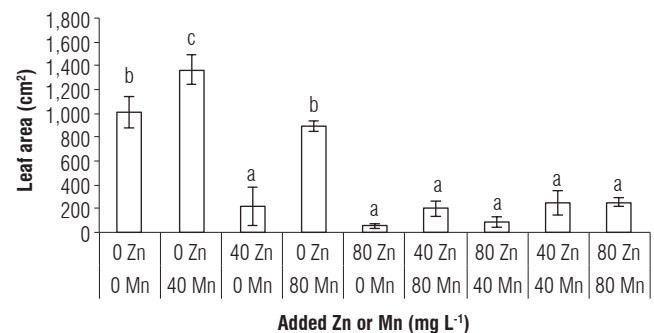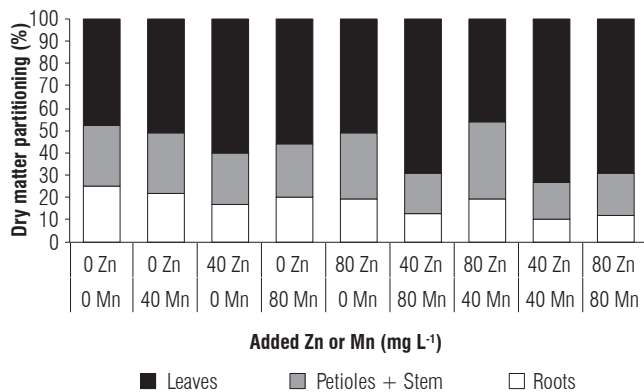

FIGURE 1. Leaf area in spinach plants (*S. oleracea* L.) grown with excess zinc and manganese in the nutrient solution. Bars above columns denote standard deviation. Means with different letters indicate significant difference according to Tukey test ($P \leq 0.01$).

Ca, Mg, Cu, Mn, Ni, Zn, or Fe; Ni with Zn, Fe, or Mn; and Cu with Ca (Rashed, 1995).

On the other hand, Lidon (2002) reports that in shoots of 15-d-old rice plants, increasing Mn concentrations triggered an antagonistic effect with the contents of Fe, Cu, and Zn, but a synergistic tendency was found thereafter. During all the experimental periods, net Mn and Cu uptake increased until the last treatment, but the absorption of Zn and Fe increased only 21 and 28 d after germination. Total shoot accumulation of these micronutrients displayed similar patterns from the 21st d onwards. After this experimental period, the translocation rates also showed a synergistic increase for all metals, but in 15-d-old rice plants an antagonism was found between Mn and Zn. It was concluded that before and after the end of the mobilization of seed reserves, rice adaptation to high Mn concentrations is associated with significant changes of micronutrient accumulation in the tissues (Lidon, 2002).

In the present work we can also observe Zn/Mn antagonism. In the results for leaf area in plants exposed simultaneously to Zn and Mn, Zn induced greater toxicity than Mn, reflected by a smaller leaf area in plants exposed to high Zn contents. Nevertheless, when both metals were added to the nutrient solution, Zn toxicity symptoms were attenuated by Mn.

Exposure of plants to high Mn content can not only reduce leaf area, but even cause defoliation, although in the present study such a reduction was not observed. In watermelon, Mn toxicity is so dramatic that it is referred to as "sudden crash" syndrome. The crop looks quite healthy and grows rapidly from transplant or sowing to flowering, or sometimes to the early fruit-filling stage, and then the older leaves suddenly wilt, dry up, and drop in a matter of days.
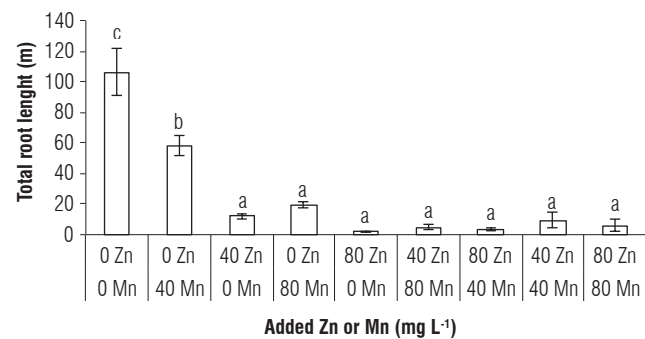
Loss of leaves reduces the ability of plants to absorb sunlight for photosynthesis, resulting in reduced fruit set, fruit size, and quality (Cooperative Extension Service, 1998).

In the present experiment, exposure of spinach plants to Zn and/or Mn changed patterns of dry matter allocation in different organs of the plant. The dry matter accumulated in leaves, in roots, and in stems + petioles showed statistically significant differences between the treatments ($P \leq 0.01$). Except for the treatment with 80 mg L^{-1} Mn + 40 mg L^{-1} Zn, the remaining treatments presented values of biomass allocation to the leaves greater than in the control plants. Even the addition of 80 mg L^{-1} Zn and 80 mg L^{-1} Mn induced 45.05% more allocation of biomass to the leaves than in the controls (Fig. 2). The addition of 80 mg L^{-1} Zn and 40 mg L^{-1} Mn + Zn 80 mg L^{-1} led to an increase of 9.24 and 29.75%, respectively, in dry matter allocation to the stem + petioles. All other treatments induced a reduction in dry matter allocated to the petioles + stem. Accumulated dry matter in the roots of treated plants was lower in all cases than that accumulated in roots of the control plants, with statistically significant results ($P \leq 0.01$).

In similar experiments, Casierra-Posada *et al.* (2010) found that broccoli plants exposed to high Zn concentrations reduced dry matter allocation to the roots in an inversely proportional relation to Zn soil concentration, as this metal principally affects roots. In the present study, in plants exposed to high concentrations of Zn and Mn together, it was found that the Mn softened the toxic effect of Zn on the roots (as it did with leaf area) due to an antagonistic relation between the two elements.

In the present study, the roots were the organ most affected by both excess Mn and Zn, as well as by the interaction of both metals, added to the nutrient solution. All treatments

FIGURE 2. Dry matter partitioning in spinach plants (*S. oleracea* L.) grown with excess zinc and manganese in the nutrient solution.

FIGURE 3. Total root length in spinach plants (*S. oleracea* L.) grown with excess zinc and manganese in the nutrient solution. Bars above columns denote standard deviation. Means with different letters indicate significant difference according to Tukey test ($P \leq 0.01$).

showed a significant effect ($P \leq 0.01$) on root length. While the addition of 40 and 80 mg L^{-1} of Mn reduced the total root length by 45.06 and 81.64%, respectively, as compared with the controls, Zn concentrations of 40 and 80 mg L^{-1} reduced root length by 88.78 and 98.07%, respectively. All mixtures of Zn and Mn together in the nutrient solution also decreased this variable, over 90% (Fig. 3).

The first visible damage in plants due to excess zinc is commonly observed on root growth (Harmens *et al.*, 1993), which corresponds with the present study's observations in spinach. Powell *et al.* (1986) reported that elongation of the longest root was much more inhibited by increasing Zn concentration in a non-tolerant (S59) cultivar than in tolerant (Merlin) red fescue plants. The mitotic index in the root meristem was reduced to a greater extent by increasing Zn concentration in S59 than in Merlin, while the cell doubling time was increased by Zn much more in S59 than in Merlin. Treatments with 0.1 and 0.2 mg Zn L^{-1} increased the length of the cell cycle by 40 and 132% respectively (compared with the zero Zn control treatment) in S59, but only by 6 and 16% respectively in Merlin. The increase in cell cycle length was due mainly to an increase in the duration of G1 in both cultivars; Zn had little effect on the duration of the other phases of the cell cycle. The growth fraction was progressively reduced by increasing Zn concentration in S59 but was increased with 0.1 mg Zn L^{-1} in Merlin and then reduced to the control level at the higher Zn concentration. Based on these results, we can infer that the reduction in root growth found in the present study is due to a reduction in cell division caused by excess Zn.

The results of the present study for water uptake, agronomic efficiency of water use, fresh weight, dry weight, and root to shoot ratio, showed statistically significant differences ($P \leq 0.01$). The addition of 40 mg L^{-1} of Mn resulted in an increase in values of these variables by 21.24; 0.43; 7.79; 22.0 and 13.71%, respectively, compared with the controls. The remaining treatments in which Zn and/or Mn was added, produced a decrease (Tab. 1). On the other hand, the specific leaf weight showed significant differences ($P \leq 0.01$) in some but not all treatments, as compared to the control.

It has been found in *Citrus volkameria,* that excess Mn is tied up by plants in the form of increased chloroplasts (Papadakis *et al.*, 2007). If such a mechanism also exists in spinach, it would explain many results in the present study. Increased chloroplasts would increase photosynthetic capacity, thus augmenting factors such as leaf area and dry weight. Roots, on the other hand, would not possess such a photosynthetic mechanism for detoxifying excess manganese, and hence would display the reduced growth seen in the present study.

Although manganese is not a common pollutant in soils, various soil conditions often present in acid and volcanic soils, or flooding conditions, can lead to Mn reduction or increase and create Mn toxicity in many natural and agricultural systems (Foy *et al.*, 1978). The deleterious effect of Mn toxicity is often observed in the shoot as stunted growth, chlorosis, crinkled leaves, and brown lesions (Marschner, 1995), although such effects were not observed in the present study. In the current study, as a result of the reduction in leaf area and root length caused by the toxicity of both metals, the plants took up less water and thus the agronomic efficiency of water use was negatively affected. In a study conducted to evaluate the nutrient composition of biomass and conductive tissue in the habanero pepper, the order of concentrations in mg plant^{-1} was: Fe> Zn> Mn> Cu at four tested growth

TABLE 1. Growth parameters in spinach plants (*S. oleracea* L.) grown with excess zinc and manganese in the nutrient solution.

Treatments		Growth measurements					
Mn	Zn	Water uptake	Agronomic efficiency of water use	Fresh weight	Dry weight	Specific leaf weight	Root to shoot ratio
(mg L^{-1})	(mg L^{-1})	(L)	(g L^{-1})	(g)	(g)	(mg cm^{-2})	
0	0	5.32 c	2.34 c	119.26 c	12.41 c	5.84 ab	3.94 c
40	0	6.45 d	2.35 c	128.77 c	15.14 d	5.70 a	4.48 c
0	40	2.03 a	1.51 abc	19.04 a	3.04 a	9.35 abc	0.97 a
80	0	4.32 b	2.26 c	83.16 b	8.98 b	5.73 a	2.55 b
0	80	1.78 a	0.60 a	5.10 a	1.08 a	10.06 bc	0.77 a
80	40	1.65 a	1.61 abc	14.81 a	2.68 a	9.51 abc	0.68 a
40	80	1.55 a	1.22 ab	7.62 a	1.87 a	11.59 c	1.06 a
40	40	2.05 a	1.55 abc	19.56 a	3.27 a	10.40 c	0.67 a
80	80	1.88 a	1.83 bc	13.59 a	3.45 a	9.43 abc	0.85 a

Means in the same column followed by the same letter are not significantly different according to Tukey test ($P \leq 0.05$).

stages, with Mn slightly exceeding Zn after 75 d of plant development. The dynamics for Zn and Mn showed an increase in the early stages of development, and declined with plant age, especially for Zn (Noh-Medina *et al.*, 2010). Based on this result we can infer that plants show a differential response to uptake of heavy metals according to the growth stage and also have a different sensitivity to each metal that may present a toxicity risk. All these factors negatively affect the amount of dry matter produced by each plant, due to the alterations caused by these metals on the agronomic efficiency of water use. On the other hand, the root to shoot ratio is affected as dry matter partitioning to the various plant organs changes.

Conclusions

In general, the addition of excess zinc was highly toxic to the plants, while excess manganese showed less drastic effects as compared to the control treatment. In some cases (leaf area and dry weight, for example), excess Mn even increased growth parameters, while with Zn such increases were limited to dry matter allocation to the stems and petioles and specific leaf weight. In some cases, the presence of excess Mn seemed to attenuate the effects of excess Zn on the plant, though this tendency was not consistent and was often anecdotal. The results of the present study are relevant for areas such as the southern Bogota plain, where horticultural production occurs near municipal waste disposal sites. The highly toxic effects of excess Zn and Mn on spinach in the present study point to the importance of monitoring heavy metal content in soil and groundwater at such sites, to assure good plant growth. In addition, the absence or subtlety of Mn effects on plants is a warning against relying solely on visual indicators to detect heavy metal presence; according to the present study, it would be possible in certain cases to obtain satisfactory yields and products of normal appearance that may in fact contain levels of heavy metals that are dangerous for human consumption.

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