Effect of Cd, Cr, Cu, Mn, Ni, Pb and Zn on seed germination and seedling growth of two lettuce cultivars (*Lactuca sativa* L.)

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Abstract

In this study, the effect of different concentrations of Cd, Cr, Cu, Mn, Ni, Pb and Zn on seed germination and seedling growth at an early stage of two lettuce cultivars (one with green and the other with red leaves) was evaluated. The inhibitory effects of these metals on germination rate, viable seedlings, shoot and root biomass, root length, seedling vigor and root tolerance index was determined. Globally, a decrease was observed in these variables with increasing concentrations of the metals. The present results indicate that seedling growth was more sensitive than germination. *Lactuca sativa* seeds were usually tolerant for all metals during the germination process and this probably occurred due to barrier effect of seed coat that prevented the metals to come in contact with the developing embryo. The inhibitory effects of these metals on seedling growth varied. In general, the presence of low Ni and Cr concentrations stimulated the growth of green-leaf lettuce seedlings. Low concentrations of Zn promoted the growth of red-leaf lettuce which is less tolerant to Cd. In this study it was verified that *Lactuca sativa* seedlings can survive in contaminated media, however, it was more sensitive to Cd and Cu and tolerant to Mn.

Keywords: heavy metals, cadmium, copper, lettuce, essential elements, non-essential elements.

Introduction

In recent years, industrialization and urbanization has led to the incorporation of pollutants, like heavy metals, in natural resources such as soil, water and air, leading to degradation of the environment. Heavy metals including Cd, Cr, Ni, Cu, Zn and Mn are important environmental pollutants that can cause toxic effects in the plants (Nagajyoti et al. 2010; Mourato et al. 2015).

Lettuce is an extensively consumed crop and is a bio-indicator species of heavy metals, as it is a living organism whose behavior and physiological state is closely correlated with the environment where it develops, so its observation sheds light on the quality and characteristics of the environment (Bagur-González et al. 2011). It is considered an accumulator of heavy metals such as Cd, Pb and Zn (Jordão et al. 2006).

Germination assays and development of seedlings in the early stages of growth are important since these initial phases will determine the yield and vigorous growth of the adult plant. Seed germination is one of the most sensitive physiological processes of the plants that are more susceptible to contamination by heavy metals especially when they come in contact with the embryo due to a lack of certain defenses. Germination is the first step in the life of a plant and it is in an adverse environment the germination process hardly occurs (Ozdener and Kutbay 2009). Inhibition of germination appears to be the first defense mechanism which seed has against to adverse environmental conditions.

The effect of heavy metals in imbibition and radicle elongation depends on its ability to penetrate the seed coat and change various physiological processes that are involved in germination. In many species the seed coat is an important defense of the plant against abiotic stresses, supporting the idea that the seed is a protected part of the life cycle of a plant (Kranner and Colville 2011).

There are plants where the germination rate is not affected, since the metal may be adsorbed by the seed coat, not reaching the embryo, leading to the exposure of the radicle (Di Salvatore et al. 2008). However, toxicity effect of heavy metals can lead to the appearance of non-viable seedlings, which can be seen by the radicle length (Li et al. 2005). This toxic effect of metals appears because there is a direct contact with the developing seedling, after radicle protrusion. Germination is affected by heavy metals (e.g. Cd and Cu) due to its intrinsic toxic effects and may be through its effects on the inhibition of water absorption in imbibition phase (Li et al. 2005; Lefèvre et al. 2009; Siddiqui et al. 2009) or impaired mobilization of seed reserves through the effects of hydrolytic enzymes (Kranner and Colville 2011).

Metals have a specific behavior associated with large variation in the morphology of seed coat which affects its permeability (Moïse et al. 2005). Although the seed coat provides some protective effect, it will rupture or became more permeable after germination. Low concentrations of heavy metals can stimulate germination and the vigor of seedlings due to increased defenses (Lefèvre et al. 2009; Bailly et al. 2008). However high concentrations can compromise future plant growth due to decreased seed vigor, loss of viability, weakened seedling and seedling death (Kranner and Colville 2011). Some studies indicate that in the presence of high concentrations of heavy metals seeds might still germinate but seedling growth can then be impaired (Ozdener and Kutbay 2009; Li et al. 2005).

The main objective of this study is to make a comprehensive comparative analysis of the effect of different concentrations of non-essential (Cd, Cr and Pb) and essential (Cu, Ni, Mn, and Zn) metals on germination and seedling growth of two cultivars of lettuce (*Lactuca sativa*), a green leaf and a red leaf one.

Materials and Methods

Germination and seedling growth assay

In this study we used seeds of two lettuce cultivars (*Lactuca sativa* L., var. capitata) with differently coloured leaves: one green-leaf type (“Golden Spring”, designated here GL) and one red-leaf type (“4 Seasons”, designated here RL).

The metals were added in different concentrations to a 10% Hoagland solution. Heavy metal salts used included: $CdCl_2 \cdot 5/2H_2O$, $CrCl_3 \cdot 6H_2O$, $Pb(NO_3)_2$, $CuSO_4 \cdot 5H_2O$, $NiCl_2 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$. For each metal the concentrations used were 5, 10, 25, 50, 100, 150, 250, 350, 500, 750 and 1000 µM. For Mn higher concentrations were also used: 2000, 5000 and 10000 µM. The wide range of concentrations chosen allowed to observe the full effect of the different metals in the studied parameters.

The experiment was conducted for 10 days after placement in a growth chamber at a temperature of 20/22 ºC dark/light, 65% humidity and 12h dark/light periods and a light intensity of 250 µmol m^{-2} s^{-1}. Germination occurred after 2 days when there was emergence of radicle. Each test was carried out in triplicate with 30 seeds per test. Seeds were sown on petri dishes with a layer of cotton covered with filter paper and they were wetted with 10% Hoagland solution without (control group) or with heavy metal solutions.

Analytical determinations

To evaluate the effect of heavy metals on germination and growth of lettuce seedlings, visible symptoms, germination rate, number of viable seedlings, biomass of shoot and root, root length, metal content, seedling vigor index and root tolerance index were determined.

Visible symptoms, germination rate and viable seedlings

Visible symptoms were observed during the assay. Germination rate (GR) was determined by counting the number of seeds germinated at the end of 2 days. The results were expressed as the percentage of seeds germinated. Viable seedlings measurement was carried out by counting the number of viable seedlings after 6 days of exposure to ensure that all seedlings had initial development.

Fresh shoot and root weight and root length

After 10 days, the seedling weight of shoot and root (g) was determined and the root length (cm) was measured. The percentage of growth inhibition in relation to the control was also calculated.

Seedling vigor index

To evaluate the toxic effect of each metal in lettuce seedlings the seedling vigor index (SVI) (Novo and Gonzalez 2014) was calculated:

$$SVI = GR\ (\%) \times root\ mean\ length\ (cm)$$

Metal content

The evaluation of the presence of each metal was determined in shoots of seedlings 10 days after exposition to the different metal concentrations. An acid digestion was carried out (*DigiPrep MS SCP Science*), by weighting around 0.3 g of dried plant material with 7 mL nitric acid (HNO_3, 65 %) and 3 mL hydrochloric acid (HCl, 37 %). Thereafter, the digested samples were analyzed by flame and electrothermal atomic absorption spectrophotometry (AAS, *Unicam Solar M*).

Statistical and multivariate analysis

A one-factor analysis of variance (ANOVA) was performed with the SPSS 20.0 (SPSS Inc.) software and the Tukey test was used to determine significant differences between the means ($p < 0.05$) and was carried out to compare germination rate, viable seedlings, fresh shoot and root weight, root length and metal content values. The experimental data obtained for the two lettuce cultivars was classified using a partitioning clustering method (PAM, Partitioning Around Medoids) with three clusters and this was performed using a correlation matrix with six normalized variables (fresh weight of shoots and roots, root length, metal uptake, germination rate and the number of viable seedlings), using the R studio software (Version 1.0.136, RStudio, Inc.).

A multiparametric approach with classification cluster analysis was carried out to evaluate the influence of the tested parameters in the classification and differentiation of the metal effect in the two lettuce cultivars. Data matrix containing the data of six standardized variables for each metal was subjected to a partitioning method and the results are shown in Figure 2.

Results

Visible symptoms

There was a general decrease in leaf size with increasing metal concentration, more pronounced after 10 days of treatment and for the higher metal concentrations (Online resources 1 and 2). There was also a reduction in root length and a darkening of the root tips.

Germination rate and viable seedlings

The germination rate of seeds from both lettuce cultivars was not affected for any metal concentration (results not shown).

As for the number of viable seedlings, the only difference observed was for the highest Cd concentration (1000 µM), where it was found a reduction of 44% in relation to the control.

Shoot and root fresh weight

The results for the fresh weight of shoots and roots, 10 days after germination for both lettuce cultivars are presented in Table 1.

Of the three non-essential metals studied, GL lettuce seedlings were more sensitive to the presence of Cd, causing a significant decrease in the fresh weight of the shoots at lower concentrations of the metal (≥ 25 μM) compared to Cr and Pb (both ≥ 250 μM), with a concomitant increase in the inhibition percentage. Of the seven metals studied, at the highest concentrations, Cd was the one that had a more pronounced effect in the inhibition of the growth of shoots (78 %) and roots (91 %). This metal also caused a negative effect on root fresh weight at lower concentrations (≥ 50 μM). Lead showed the same pattern but at higher concentrations (≥ 150 μM) and Cr also caused a decrease in root fresh weight at still higher concentrations (1000 μM) but caused growth stimulation of roots exposed to 50, 100 and 150 μM.

Red-leaf lettuce seedlings were also shown to be less tolerant to Cd manifesting its effect as a reduction of fresh weight of shoots (≥ 5 μM) and roots (≥ 50 μM) at lower concentrations when compared with other metals. The inhibition percentage of growth was also higher in RL lettuce seedlings compared to GL lettuce. This indicates a greater sensitivity of RL lettuce to Cd. Regarding other metals, Pb and Cr showed a very similar negative effect on the fresh weight of shoots and roots.

Regarding the essential metals, Cu, Ni and Zn had a similar effect in decreasing shoot fresh weight of GL lettuce, at concentrations of 50 μM and higher. However, Cu had a higher percentage of growth inhibition (83 %) followed by Ni (73 %) and Zn (63 %), at the higher metal concentration studied. Nickel also caused growth stimulation at lower concentrations of metal (10 μM). Copper was the only essential metal which caused a decrease in GL root fresh weight for all concentrations studied, even the lowest one of 5 μM. The roots of this cultivar had a higher sensitivity to this metal in comparison to shoots, with a maximum growth inhibition of 92 %. The presence of 10 μM Ni also stimulated root growth, but at concentrations of 25 μM and higher the opposite effect was detected. This confirms the increase in shoot weight described above. Zinc and Mn were the essential elements that had a lower toxic effect on roots.

In RL lettuce the effect of Cu, Ni and Zn in shoot biomass was very similar. Exposure to Cu reduced biomass at lower concentrations (≥ 25 μM) than Ni and Zn (≥ 50 μM). Manganese only expressed negative effect for very high concentrations (≥ 500 μM). The adverse effect on root fresh weight for [Cu] and [Ni] ≥ 50 μM was similar but the percentage of inhibition was greater for Cu (92 %) than Ni (78 %) at 1000 μM. Zinc promoted growth at lower concentrations (5 μM) expressing a negative effect at higher concentrations.

Root Length

A decrease of the root fresh weight was accompanied by a reduction in root length due to the presence of heavy metals (Table 2). Root length decreased with exposure to increasing concentrations of the metals and was more pronounced for the highest concentrations tested. Both cultivars had the same pattern for root length decrease. The strongest effects were observed for Cd, followed by Cu and Ni. Manganese was the element that less affected root length.

Metal Uptake

There was an accumulation of all the metals studied in the shoots of both lettuce cultivars seedlings that increased with higher concentration of all metals (Figures 1A and 1B). For both cultivars, at the two lowest metal concentrations (5 and 10 μM) the three metals most absorbed by the plants were Mn, Zn and Cd, always at concentrations higher than 100 mg/kg DW (all the other metals were at concentrations lower than this value). For metal concentrations between 25 and 100 μM, the elements that accumulated more are Cd, Ni, Zn and Mn, with Pb also reaching almost the same levels. For the highest metal concentrations, Cd was the element that accumulated most (up to almost 16000 mg/kg in GL lettuce), followed by Mn and Pb. Chromium was, by far, the element least absorbed by the seedlings.

Seedling Vigor

Seedling vigor generally decreased with the concentration of applied metal (Table 3). In relation to the non-essential metals, Cd had a more pronounced effect, with a 50% reduction in SVI in relation to the control from 25 and 50 μM, for GL and RL lettuce respectively. For both Pb and Cr the results were similar with significant reductions in SVI after 250 μM (350 μM for Pb in GL). Thus, Cd had clearly a more pronounced effect in seed development than Pb or Cr. In relation to the essential metals, SVI was more affected by Cu, followed by Ni, Zn and Mn and the effect were similar for both cultivars. For the latter only at the very high concentration of 2000 μM and higher was detected the 50% reduction in SVI in relation to the control.

Discussion

A general decrease in leaf size was detected with increased metal concentration, for all the metals studied, as can be seen in Online Resources 1 and 2 for GL and RL lettuce cultivars, respectively, after 10 days of treatment. The smaller leaves had a more intense green color, probably because chlorophyll became more concentrated as the leaf area decreased. In lettuce seedlings exposed to the higher concentrations of Cd and Cu, leaf chlorosis and browning was apparent, due to the destruction of chlorophyll molecules and/or its reduced synthesis (Nagajyoti et al. 2010). It can also be hypothesized that the excess levels of the metals under study affected the uptake of essential elements, like Fe and Mg, thus affecting chlorophyll production and stability.

The toxic effect of the metals was much more noticeable in the roots of both lettuce cultivars with a gradual darkening of the roots (starting at the tips) with increasing metal concentration. Similar effects have been reported in different plants (Scoccianti et al. 2006; Gajewska and Sklodowska 2010) .

Exposure to Cr (≥ 250 μM), Pb (≥ 250 μM), Ni (GL:≥ 100 μM;RL:≥ 150 μM) and Zn (≥ 100 μM) led to the appearance of visible signs of toxicity at higher concentrations. Lettuce seedlings demonstrated to be more tolerant to Mn and lower concentrations of this metal had an apparent stimulation of growth (5-250 μM).

The ability to germinate in an environment contaminated with metals can be indicative of seed tolerance. There was no significant difference in germination rate, compared to the control in all metal-contaminated solutions for both lettuce cultivars (results not shown). This was probably due to the barrier effect of the seed coat protecting the developing embryo and also due to the fact that the applied stress does not affect the process of cell elongation responsible for the protrusion of the rootlet through the seed coat (the indicator used to measure germination rate) (Di Salvatore et al. 2008). As the seed coat is composed of one or more layers of thick-walled cells, usually impregnated with waxes and fats, they can absorb heavy metals and avoid their contact with the embryo inside. If the metals cannot cross the seed coat then the metabolic processes that occur in germination are not affected. Ozdener and Kutbay (2009) reported that the level of tolerance was higher in seed germination than in the further development of *E. sativa* seedlings and this was also observed for lettuce seedlings in the present work.

The fact that these metals have no impact on the germination rate, but have later in seedling development, suggests that lettuce seed coat has an important role in the selective permeability of different metals. In this case, the effect of metals on germination is not related to the inhibition of imbibition and the consequent absorption of water and mobilization of nutrients by the seed as referred by Kranner and Colville (2011) since there is this barrier effect.

After exposure of the radicle, there is direct contact with contaminant. However, there were no significant differences in the number of viable seedlings compared to the control with exposure to different metals except for the highest concentration of Cd, 1000 μM (results not shown). Munzuroglu and Geckil (2002) referred that an excess of metal concentration can result in an abnormal germination, the coating is ripped by the radicle but its subsequent development does not occur. This may be an indicator of seedling's sensitivity to the metal present in the media.

Di Salvatore et al. (2008) obtained a similar result to the present work in different plant species (lettuce, broccoli, tomato and radish) exposed to Cd, Ni, Cu and Pb, referring that no significant differences in germination rate was observed in the concentrations used (0-1024 μM). Marquez-Garcia et al. (2013) found that different concentrations of Cu, Mn, Ni and Zn (10-2000 μM) did not affect the germination rate of *Atriplex halimus*, with no significant differences compared to the control. On the other hand, there are several reported cases of germination rates decreasing with increasing levels of heavy metals (Sfaxi-Bousbih et al. 2010; Lamhamdi et al. 2011; S. Liu et al. 2012).

These results lead to the conclusion that seed germination under heavy metal stress is highly dependent on plant species and the two lettuce cultivars used in this work have seeds that are highly resistant to different heavy metals under relatively high concentrations.

A general decrease in fresh weight of both cultivars was measured with increasing metal concentration (Table 1). Manganese only expressed a negative effect at very high concentrations (≥ 500 μM) showing the low level of toxicity of this element to lettuce seedlings. However, there was an observed stimulation of growth with Cr and Ni for GL and with Zn for RL. This effect was explained by Bailly et al. (2008) indicating that lower concentrations of metals can stimulate seedling growth due to the increased level of oxidative stress, which may cause an increase in ROS signaling. A similar explanation of this stimulus is also given by Kranner and Colville (2011) due to the activation of the defense mechanisms of young plants. Although lettuce has been reported as being very sensitive to Ni, a stimulation of root growth by low concentrations of this metal has been observed (Carlson et al. 1991).

The observed decreased in lettuce shoot and root biomass with higher metal concentrations is a common toxic effect and has been verified by several authors. Jordão et al. (2006) reported reduced lettuce yields in plants growing in vermicompost with added Cu, Ni and Zn.

Gajewska and Sklodowska (2010) observed a decrease in the growth of shoots and roots in wheat seedlings exposed to 75 µM of Cu while, in another study, these authors found that exposure to 50 and 100 µM of Ni after 7 days led to a significant decrease in the fresh weight of shoots and roots of wheat seedlings compared to the control (Gajewska et al. 2012).

The growth of shoots and roots is influenced by the presence of increasing concentrations of metal and for the different metals studied different concentration thresholds were observed before fresh weights were significantly decreased. For both cultivars studied in this work, root growth was more inhibited than shoot growth. This occurs because in media with high concentration of metals, the reduction of root growth is associated with direct contact with the contaminant. This is in part due to an inability of the roots to absorb water and nutrients from the environment, which will also impact the development of shoots.

The type of metal present in the medium is decisive in young plant response. Green-leaf lettuce is less tolerant to the presence of Cd compared to Pb or Cr. Red-leaf lettuce shoots was most sensitive to Cd. The presence of Pb and Cr led to a very similar behavior in the biomass of root for both cultivars, except that for GL lettuce the presence of low Cr concentrations stimulated root growth.

Regarding essential elements, Cu was the one that most negatively affected the fresh weight of lettuce seedlings shoots and roots. Green-leaf lettuce was more sensitive to its presence, especially the roots. A toxic level of metal affects growth of the seedlings due to the negative effect on various metabolic processes, resulting in low elasticity of the cell walls, inhibiting cell division, as well as suppressing the activity of hydrolytic enzymes (Pandey and Sharma (2002).

Root analysis, like its length, is widely used to assess the level of toxicity of heavy metals and the inhibition of root length is considered an early evidence of its toxic effects (Visioli et al. 2014).. In the present study, a decrease in root length was detected and was more pronounced for the highest metal concentrations (Table 2). These results are in agreement with those obtained by other authors in different plants while evaluating Cd and Zn (Lefèvre et al. 2009), Cu, Cd, Ni, Pb and Zn (Ozdener and Kutbay 2009) or Cd (S. Liu et al. 2012).

There were significant differences in the root length of lettuce seedlings from both cultivars exposed to Cd compared to the control at concentrations as low as 5 μM. This reinforces what has been said previously showing a low tolerance of lettuce seedlings to this contaminant. Root length decrease due to the presence of Cd has also been reported in several plants like wheat (X. Liu et al. 2007), rocket (Ozdener and Kutbay 2009) and pea (Siddiqui et al. 2009). An increased Cr concentration promoted a significant decrease in root length of lettuce seedlings at concentrations ≥ 150 μM. Similar results caused by Cr were obtained by Scoccianti et al. (2006) . Exposure to Pb showed that there are significant differences in both lettuce cultivars at concentrations ≥ 50 μM compared to the control. This tendency was also observed in wheat (Lamhamdi et al. 2011) and rocket (Ozdener and Kutbay 2009).

For Cu and Ni there was a significant decrease in root length from 25 μM for both lettuce cultivars. Inhibition of root growth due to the presence of Cu is a frequently reported result in several plant species (Mihoub et al. 2005; Ozdener and Kutbay 2009). Although there was a small increase in GL root fresh weight at a concentration of 10 μM Ni, no significant effect on root growth was detected. This shows that root morphology changed, with shorter and more branched main roots formed at this low Ni concentration.

Regarding the effect of Zn, a significant decrease in root length of both lettuce cultivars from 50 μM of Zn compared to the control was observed. Marquez-Garcia et al. (2013) also reported that Zn concentrations between 250 and 2000 μM caused a significant decrease in root length of two halophyte species.

For Mn, only at concentrations higher than 1000 μM, was a significant reduction in root length detected.

The type of metal affects the response of root against its toxic effects. Cadmium and Cu caused a higher inhibition in root length which is consistent with the results obtained previously. Manganese was the metal which showed less effect on the length of the lettuce root and it was necessary to increase the concentration to very high values to obtain a similar result.

This study also confirmed that root growth was much more sensitive to the presence of metals than the germination process (Di Salvatore et al. 2008; Marichali et al. 2014), with different intensities detected for different metals. As heavy metals affect different metabolic processes (Mourato et al. 2012), the decrease in root length may be related to the interference of the metals with cell division, cell proliferation and elongation (Michael and Krishnaswamy 2011) and loss of root cell viability (Finger-Teixeira et al. 2010). It can also affect the storage of nutrients in the formation of the embryo which can change the mobilization of nutrients (Sfaxi-Bousbih et al. 2010; Karmous et al. 2011), the activity of amylases (Sfaxi-Bousbih et al. 2010; Kranner and Colville 2011) and proteases (Mihoub et al. 2005; Karmous et al. 2011). The accumulation of metals in the root can thus reduce the mitotic rate in meristematic areas, especially by blocking the metaphase, leading to decreased length.

As expected, there was an increase in the concentration of all metals in the shoots of both lettuce cultivars seedlings with increasing metal concentration in the solution (Figures 1A and 1B). At concentrations between 150 and 500 μM for GL and 100 and 500 μM for RL, the metal most absorbed is Mn. Even with high concentrations of this metal in the plant, the toxic effects, as described above, are still mild compared to other elements like Cd, confirming the low toxicity of Mn in these plants. Thus, lettuce seedlings can absorb large amounts of this essential element without showing toxic effects. At the two highest concentrations (750 and 1000 μM) Cd was by far the metal present at a highest concentration in the shoots. This shows the high mobility of this element in the plant and is also a result of the complete breakdown of the plant defense system. Chromium levels in the plants remained very low for all concentrations studied, with values lower than 400 mg/kg DW even at 1000 μM, showing that the uptake of this element is highly restricted. Even so, these values are higher than those reported for other plants, like in 15-day-old celery seedlings where plants growing under 1000 μM Cr only accumulated around 90 mg/kg in the leaves (Scoccianti et al. 2006). Even with this restriction in Cr uptake, its toxic effects are still considerable as shown above. It is also notable that Cu can induce relatively severe toxic effects but showed lower uptake levels than other metals like Ni and Pb. While Pb is reported not to be very mobile in plants there was a strong increase in this element uptake at the two highest concentrations studied.

Seedling vigor (SVI) is an important parameter to evaluate the potential for development of normal seedlings under the conditions studied. A decrease in seedling vigor with metal concentration was detected in the present study as presented in Table 3. The strongest effects were due to Cd, Cu and Ni, in this order, for both cultivars. The effect of Cr, Pb and Zn on this parameter was similar (slightly higher effect of Cr on RL lettuce).

In their study with wheat, sunflower and canola Moosavi et al. (2012) observed a strong effect of Cd, Pb and Zn on the vigor index, but with smaller differences between the metals than in our study, probably due to different sensitivities of the studied plant species.

As can be seen in Figure 2, with Cd treatments, one class was formed for individuals exposed to Cd with the highest concentration (1000 μM), another grouped individuals with lower concentrations (0-50 µM) and yet another with slightly higher concentrations (100-500 µM). This trend also occurs for Cu exposure where there is also a very distinct class with individuals submitted to the two higher concentrations (500-1000 µM) and other two classes with individuals with control and lower concentrations (0-100 µM) and another with intermediate concentrations of Cu (150-350). For Cr contamination a distinct class was also identified for concentrations higher than 250 µM, showing that the main toxic effects were globally more intense at these higher concentrations. For Pb and Zn the results were very similar, with a grouping for the control and the lowest concentrations, and two others at the medium and higher metal concentrations, showing that some toxic effects were already apparent at those medium concentrations. With Ni, the effect is similar, with a different grouping where the strongest toxic effects were observed for concentrations above 500 µM. For Mn, there is a clearly separated grouping for concentrations of 1000 µM and above as below this value, the toxic effects were much less visible than for other metals. This separation can be explained by the sensitivity that lettuce seedlings presented mainly to Cd and Cu, where the higher concentrations of these elements had a noticeable toxic effect, and thus a completely separate group with the toxic effects at the two highest concentrations was observed.

Conclusions

Lettuce seedlings germinated under all tested concentrations of Cd, Cr, Pb, Cu, Ni, Zn and Mn (between 5 and 1000 µM, except for Mn that went up to 10,000 µM). Lettuce seed coat provided a barrier to the entry of different metals into embryo in both cultivars, thus avoiding deleterious effects on the germination process. For this reason, the germination process is not adequate to be used as the sole indicator of the toxicity of a metal, either essential or non-essential.

The number of viable seedlings in the early days of development also showed a certain tolerance of lettuce seedlings growing in contaminated media except for exposure to Cd.

Further growth and development of lettuce seedlings had a higher sensitivity to the presence of metals, than the germination process. Toxicity caused by the studied metals led to visible adverse symptoms in seedlings (inhibiting the growth of seedlings, root browning, reduced leaf size and root appearance of chlorosis and necrosis), decreased shoot and root biomass, root length, seedling vigor and tolerance index. This may suggest that a reduction in lettuce seedling tolerance is related to adverse effects caused by the toxicity of metals, which can trigger oxidative stress in this early stage of development. Roots were more affected by metal toxicity than shoots because they are in direct contact with the contaminant and its toxicity probably affect the absorption of water and nutrients.

The ability of lettuce seedlings to tolerate the increasing toxicity of each metal depends on the type of metal present and plant cultivar. In general, it can be referred that lower concentrations of Ni and Cr stimulated the growth of GL lettuce seedlings which is more sensitive to the presence of Cu. Low Zn concentrations also stimulated the growth of red lettuce and this cultivar shows less tolerance to Cd. In general, the effect of the presence of non-essential metals can be classified as: Cd >Pb >Cr and essential metals as: Cu >Ni >Zn >Mn. Although lettuce seedlings were able to limit the uptake of Cr, it still caused severe toxic effects. Cadmium was shown the most mobile element in the plant with very high uptake values. Through this study we generally observed that lettuce seedlings can survive in environments contaminated with various concentrations of metals proving to be more sensitive to the presence of Cd and Cu and more tolerant to the presence of Mn.

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Table 1 Fresh weight percentage changes in relation to the control (considered as 100%) of shoots and roots of GL (A) and RL (B) lettuce seedlings exposed to increasing concentrations of Cd, Cr, Cu, Mn, Ni, Pb and Zn. Shaded values indicate significant decreases while **bold** values indicate significant increases in relation to the control (Tukey test, $p<0.05$)

A Green leaf lettuce

[Element]	Roots (%)							Shoots (%)						
(µM)	Cd	Cr	Cu	Mn	Ni	Pb	Zn	Cd	Cr	Cu	Mn	Ni	Pb	Zn
5	95.5	113.8	75.9	92.4	96.7	81.1	97.1	90.2	110.8	92.7	100.5	99.0	96.2	91.1
10	97.5	114.1	77.5	90.3	**130.4**	84.1	108.2	91.2	108.4	98.3	102.7	**117.6**	100.8	87.1
25	79.4	111.2	62.2	85.1	73.4	79.5	104.2	79.5	107.5	88.4	101.0	93.7	94.4	89.0
50	63.1	**156.3**	53.5	85.2	71.8	80.4	98.9	64.4	110.7	70.4	108.0	72.9	88.9	71.6
100	49.2	**175.9**	39.1	83.6	54.3	82.4	80.4	53.8	88.2	59.9	122.6	59.8	89.4	67.8
150	27.0	**157.1**	34.7	88.4	51.7	71.1	60.6	44.7	88.1	54.4	108.0	55.4	81.5	56.2
250	20.9	134.6	20.2	85.4	40.0	52.7	52.9	38.5	67.8	37.4	103.8	48.2	67.7	51.2
350	16.2	117.2	17.1	66.3	31.6	47.7	48.0	32.8	62.3	30.4	94.5	42.1	51.1	48.3
500	32.0	85.8	10.2	68.0	23.5	29.6	48.0	33.8	55.8	26.0	81.6	37.1	41.5	44.7
750	16.9	71.5	8.4	61.9	21.9	17.3	33.5	27.7	49.3	26.4	69.4	30.9	31.9	35.9
1000	9.3	41.6	7.9	53.0	18.7	18.7	32.1	22.0	37.0	16.6	67.5	26.9	27.6	37.1
2000	-	-	-	33.8	-	-	-	-	-	-	47.9	-	-	-
5000	-	-	-	18.5	-	-	-	-	-	-	29.9	-	-	-
10000	-	-	-	18.8	-	-	-	-	-	-	23.6	-	-	-

B Red leaf lettuce

[Element]	Roots (%)							Shoots (%)						
(µM)	Cd	Cr	Cu	Mn	Ni	Pb	Zn	Cd	Cr	Cu	Mn	Ni	Pb	Zn
5	106.3	90.3	96.3	133.6	101.0	91.8	**133.5**	81.4	94.1	89.0	97.9	104.5	96.5	118.2
10	94.0	104.5	100.4	115.1	104.6	93.4	98.7	76.2	106.2	89.8	98.1	96.7	103.9	108.1
25	83.7	110.5	90.7	135.7	94.8	85.3	101.8	66.4	110.2	75.5	116.1	88.4	103.1	101.2
50	60.6	97.0	75.4	105.0	69.6	107.5	104.6	59.9	102.9	53.3	100.4	69.8	112.3	80.8
100	39.2	109.6	52.6	113.1	61.8	85.4	86.1	51.5	98.8	48.9	102.3	58.0	91.3	69.4
150	30.2	94.5	34.7	118.4	55.4	81.1	84.8	39.1	76.7	37.1	102.8	50.9	76.9	57.6
250	21.1	69.7	27.8	101.1	39.3	60.2	87.8	32.6	58.6	30.1	90.4	42.4	75.3	51.2
350	16.8	67.7	17.4	102.6	36.2	49.6	73.5	27.3	53.3	24.5	86.2	42.8	58.5	49.8
500	11.0	47.6	13.5	93.6	32.3	34.6	57.9	19.7	46.6	23.5	83.9	39.8	52.2	42.0

750	11.2	37.8	12.5	91.0	22.5	28.8	41.7	22.5	38.8	22.5	66.8	33.8	38.6	35.2
1000	8.3	24.6	8.4	64.3	21.9	25.5	30.8	19.2	33.3	16.7	61.8	29.8	38.9	34.3
2000	-	-	-	48.5	-	-	-	-	-	-	56.1	-	-	-
5000	-	-	-	29.8	-	-	-	-	-	-	44.7	-	-	-
10000	-	-	-	19.3	-	-	-	-	-	-	36.4	-	-	-

Table 2 Root length percentage changes in relation to the control (considered as 100%) of GL and RL lettuce seedlings exposed to increasing concentrations of Cd, Cr, Cu, Mn, Ni, Pb and Zn. Shaded values indicate a significant decrease in root length in relation to the control (Tukey test, $p<0.05$)

[Element]	Green leaf lettuce							Red leaf lettuce						
(μM)	Cd	Cr	Cu	Mn	Ni	Pb	Zn	Cd	Cr	Cu	Mn	Ni	Pb	Zn
5	76.9	104.9	95.3	102.5	101.0	97.1	100.5	78.5	91.9	96.9	109.5	100.4	94.3	103.9
10	70.4	107.0	95.2	104.3	107.2	95.5	104.2	68.7	100.6	95.1	107.7	98.9	93.6	99.6
25	35.1	104.8	61.9	105.1	76.7	93.1	98.9	61.3	98.8	70.5	110.4	71.4	93.4	97.2
50	23.0	98.8	45.3	104.6	63.4	86.5	84.5	28.8	94.8	45.1	108.1	56.2	85.9	80.0
100	9.2	77.2	36.2	106.0	33.9	79.0	69.7	19.1	92.4	33.2	109.9	45.3	77.6	70.1
150	4.9	67.6	24.0	106.0	29.6	68.7	57.6	15.4	64.5	15.1	112.1	40.8	67.6	57.9
250	2.9	58.2	7.9	98.3	14.1	37.6	48.9	3.8	35.7	8.0	109.6	17.0	27.8	35.4
350	3.4	33.9	5.2	99.6	11.7	24.9	29.4	2.9	28.5	2.9	99.6	10.8	19.9	28.7
500	3.4	23.2	3.6	97.1	11.1	15.6	27.3	1.4	22.0	3.6	99.2	8.7	15.0	21.9
750	2.3	20.6	1.8	86.6	10.5	6.1	18.5	2.0	12.3	3.1	90.2	5.1	9.4	16.3
1000	0.8	18.9	1.3	77.6	8.5	4.7	15.7	1.8	9.5	3.0	76.7	4.5	6.8	13.5
2000	-	-	-	38.0	-	-	-	-	-	-	48.1	-	-	-
5000	-	-	-	15.8	-	-	-	-	-	-	21.1	-	-	-
10000	-	-	-	10.5	-	-	-	-	-	-	9.11	-	-	-

Table 3 Seed Vigor Index for both lettuce cultivars, GL and RL, under different metal concentrations. Shaded values correspond to at least a 50% decrease in SVI in relation to the control, presented in the first line, 0 µM (Tukey test, $p<0.05$)

Element	Green leaf lettuce							Red leaf lett			
concentration (µM)	Cd	Cr	Cu	Mn	Ni	Pb	Zn	Cd	Cr	Cu	Mn
0	873.2	827.9	904.7	873.5	785.5	872.0	907.5	1051.2	1031.8	1039.5	1012.6
5	687.6	868.6	871.6	905.7	811.9	846.8	932.9	834.4	948.0	1030.0	1115.5
10	585.1	886.0	861.0	931.9	862.2	832.7	967.0	721.9	1038.2	999.8	1109.5
25	284.2	867.4	566.5	928.1	623.9	812.1	908.0	652.1	1008.2	750.0	1124.5
50	200.6	818.3	414.7	934.6	504.0	754.2	775.4	303.1	977.7	474.3	1088.3
100	79.1	646.1	327.4	915.7	263.0	696.3	647.1	203.5	953.4	345.0	1131.5
150	39.2	559.3	217.4	946.7	238.2	605.8	540.4	163.3	665.2	160.6	1141.1
250	24.1	487.3	72.6	878.3	112.3	331.4	459.0	40.4	367.9	84.9	1128.4
350	29.0	283.7	47.9	890.0	94.5	216.8	273.3	30.0	290.3	29.7	1014.2
500	29.7	194.1	32.1	852.9	83.9	137.4	244.8	14.6	214.8	36.7	1022.0
750	20.5	172.9	16.0	764.9	82.5	54.2	171.8	21.0	125.7	33.0	918.2
1000	7.1	156.3	11.6	658.9	68.7	40.4	145.8	19.1	97.3	31.7	789.6
2000	-	-	-	305.4	-	-	-	-	-	-	473.8
5000	-	-	-	133.7	-	-	-	-	-	-	217.1
10000	-	-	-	85.2	-	-	-	-	-	-	92.8

Figure legends

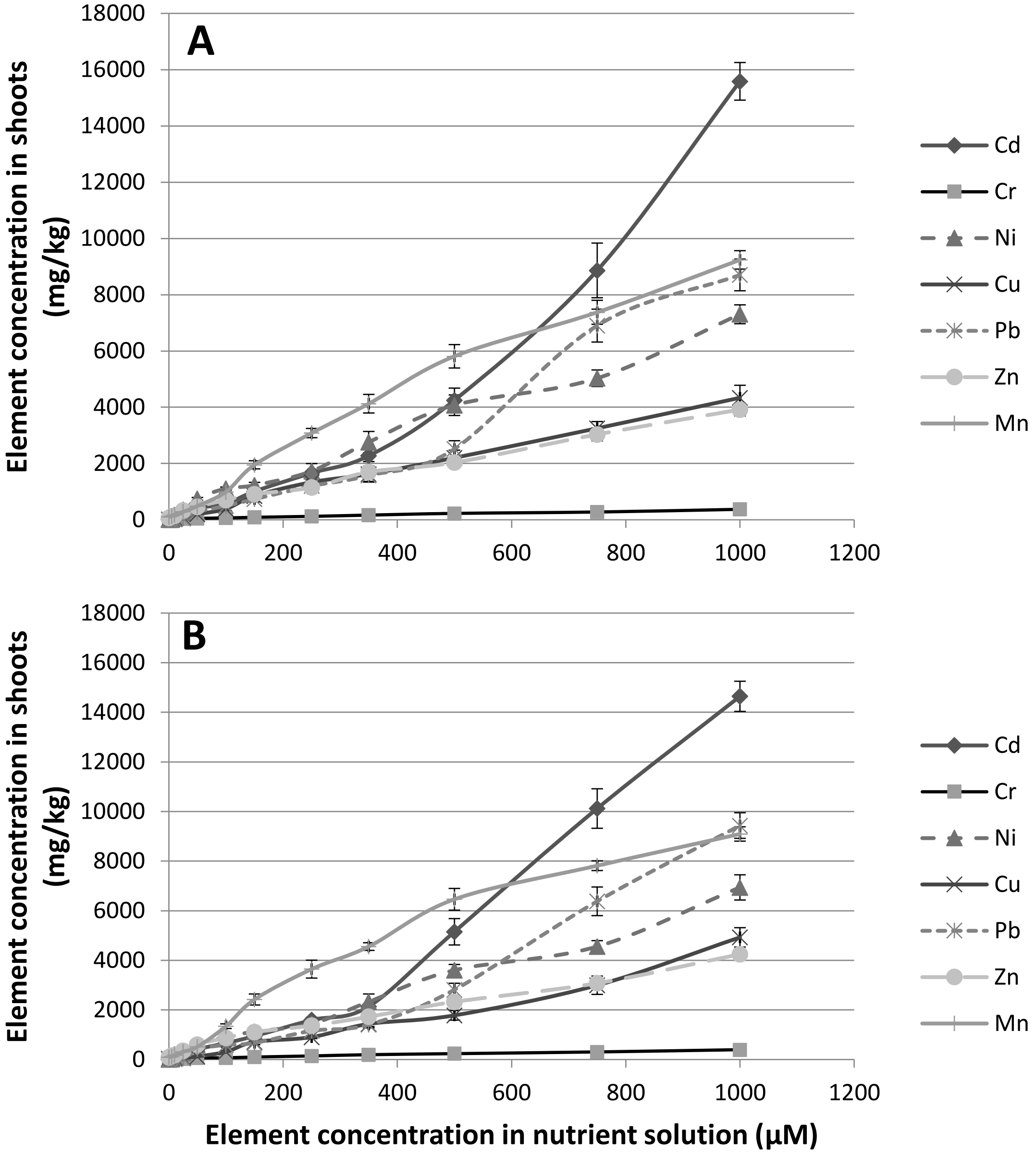
Fig. 1 Uptake of metals in shoots of (A) GL and (B) RL lettuce seedlings subjected to different concentrations of the same metals in the nutrient solution, 10 days after exposition to the different metals

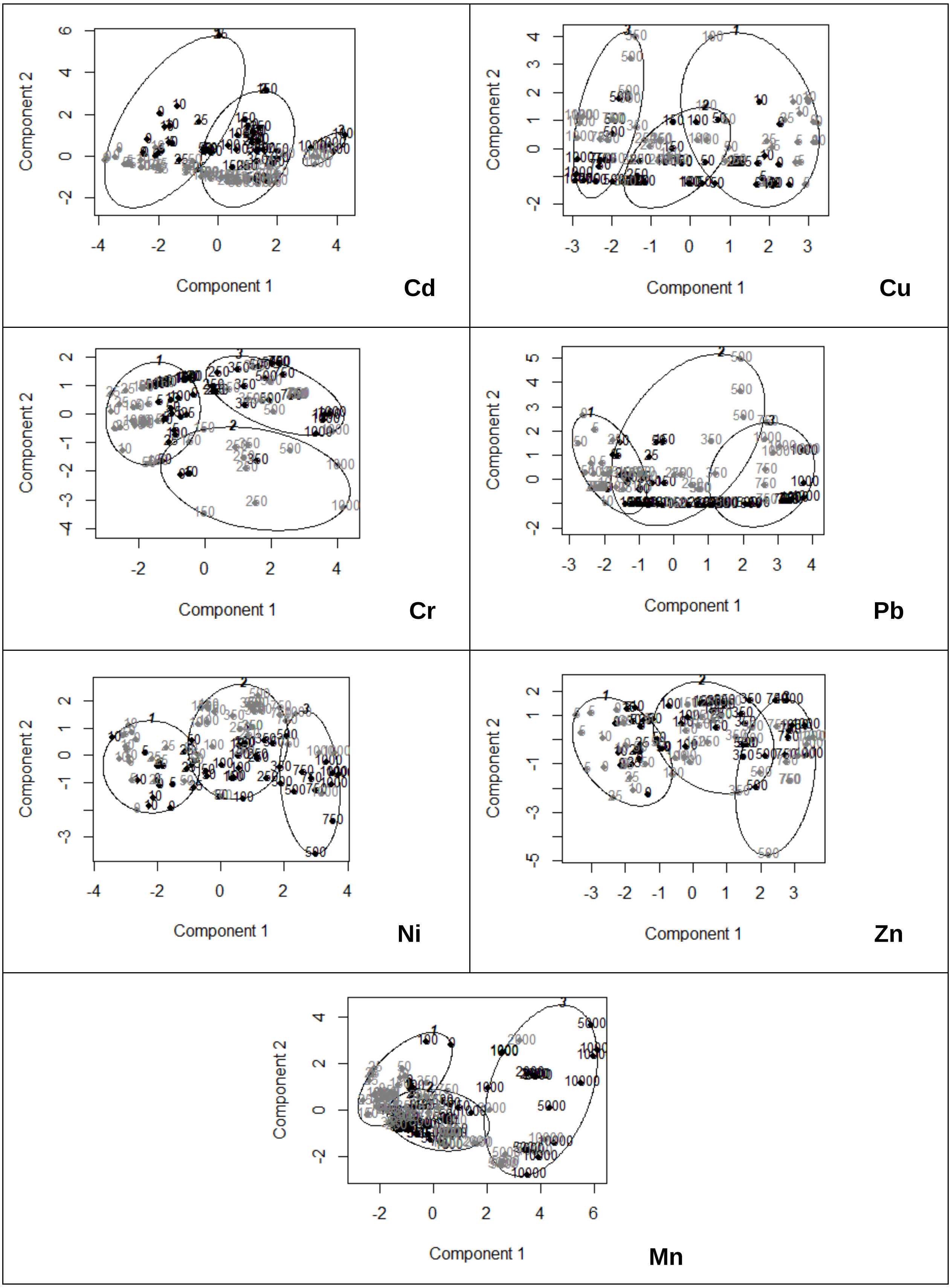
Fig. 2 Clusters obtained by PAM method for the effects of the 7 metals under study (Cd, Cu, Cr, Pb, Zn, Ni and Mn). **Black individuals** – green-leaf type lettuce seedlings (GL); **Grey individuals** – red-leaf type lettuce seedlings (RL); Individuals are represented by the concentration applied.

PAM was performed with three clusters and using a correlation matrix with six normalized variables (fresh weight of shoots and roots, root length, metal uptake, germination rate and the number of viable seedlings)

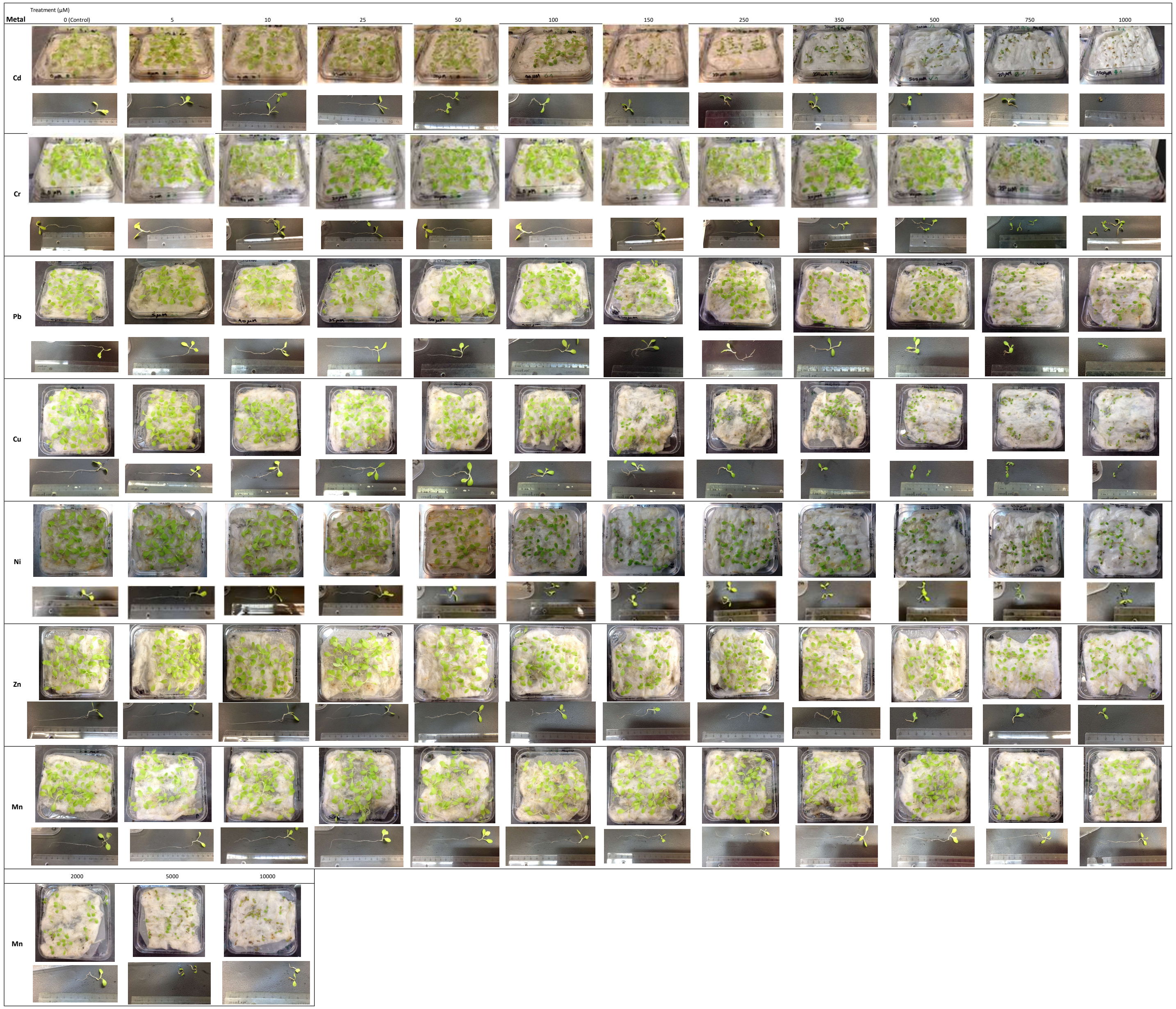
Online resource 1 Photographic record of green-leaf lettuce growing for 10 days under different heavy metal concentrations

Online resource 2 Photographic record of red-leaf lettuce growing for 10 days under different heavy metal concentrations

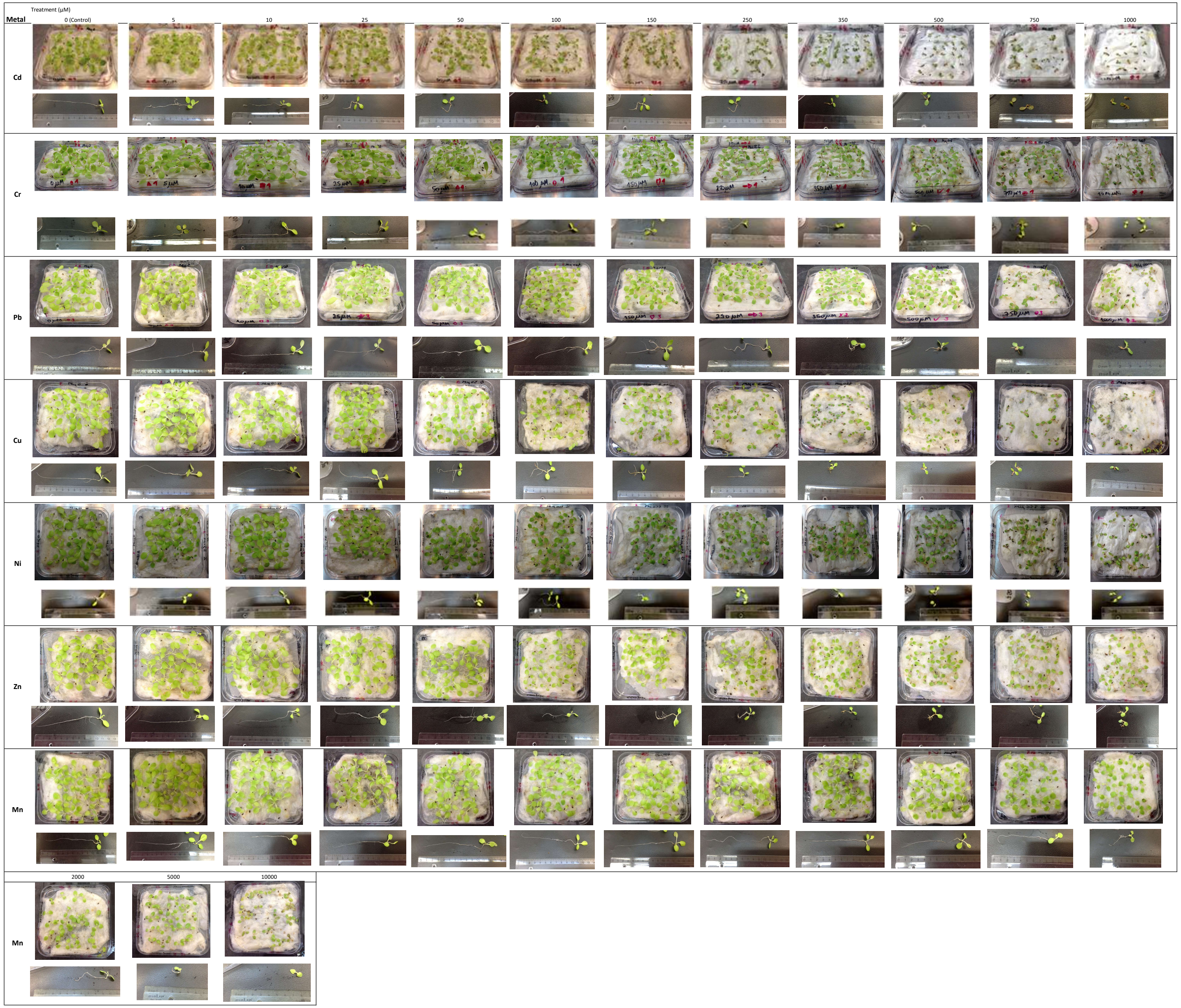

A
B
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
200
400
600
800
1000
1200
Element concentration in shoots (mg/kg)
Element concentration in nutrient solution (µM)
Cd
Cr
Ni
Cu
Pb
Zn
Mn



Component 2
Component 1
Cd
Component 2
Component 1
Cu
Component 2
Component 1
Cr
Component 2
Component 1
Pb
Component 2
Component 1
Ni
Component 2
Component 1
Zn
Component 2
Component 1
Mn



Treatment (μM)
Metal
0 (Control)
5
10
25
50
100
150
250
350
500
750
1000
Cd
Cr
Pb
Cu
Ni
Zn
Mn
2000
5000
10000
Mn


Metal
Treatment (µM)
0 (Control)
5
10
25
50
100
150
250
350
500
750
1000
Cd
Cr
Pb
Cu
Ni
Zn
Mn
2000
5000
10000
Mn