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Assessment of the root system of *Brassica juncea* (L.) czern. and *Bidens pilosa* L. exposed to lead polluted soils using rhizobox systems

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ABSTRACT

The purpose of this study was to compare the behavior of the root system of one of the most frequently cited species in phytoremediation Indian mustard [*Brassica juncea* (L.) Czern.] and a representative perennial herb (*Bidens pilosa* L.) native of Argentina, for different concentrations of lead in soils through chemical and visualization techniques of the rhizosphere. Lead polluted soils from the vicinity of a lead recycling plant in the locality of Bouwer, were used in juxtaposed rhizobox systems planted with seedlings of *B. juncea* and *B. pilosa* with homogeneous and heterogeneous soil treatments. Root development, pH changes in the rhizosphere, dry weight biomass, lead content of root and aerial parts and potential extraction of lead by rhizosphere exudates were determined. In both species lead was mainly accumulated in roots. However, although *B. juncea* accumulated more lead than *B. pilosa* at elevated concentrations in soils, the latter achieved greater root and aerial development. No changes in the pH of the rhizosphere associated to lead were observed, despite different extractive potentials of lead in the exudates of the species analyzed. Our results indicated that Indian mustard did not behave as a hyperaccumulator in the conditions of the present study.

KEYWORDS

rhizosphere; Pb; phytoremediation; rhizobox; *B. juncea*; *B. pilosa*

Introduction

Contamination of soils with heavy metals is nowadays considered to be one of the most serious environmental problems, because the sustainability of humankind is difficult without soil under optimal conditions (McGrath, Zhao, and Lombi 2002; Rodríguez *et al.* 2007; Rajmohan *et al.* 2014). Therefore, heavy metals have been the subject of numerous investigations in relation to their harmful effects on human health (Lippmann 1990; Kabata-Pendias and Adriano 1995; Alloway 1995; McLaughlin, Smolders, and Merckx 1998; Wang *et al.* 2003; Moiseenko *et al.* 2006), with the remediation of heavy metal polluted soils representing a technological challenge for both industries and governments. However, traditional remediation techniques based on engineering, which generally remove the contaminated soil and then stabilize it physically and chemically before returning the treated soil to the resorted site, have the disadvantage of reducing or even losing the biological soil functionality (Ghosh and Singh 2005). Consequently, in recent years, phytoremediation studies have received increasing attention as this technique provides an alternative environmentally friendly method compared to conventional ones, which uses the potential of plants to transform or remove contaminants through processes such as rhizofiltration, phytostabilization, phytoextraction, phytovolatilization, and phytotransformation (Dahmani-Muller *et al.* 2000; McIntyre 2003; Ghosh and Singh 2005; Kidd *et al.* 2009; Gunawardana, Singhal, and Johnson 2011).

The mechanisms of heavy metal tolerance vary among plant species and are determined by the metal, efficiency of absorption, translocation, and sequestration (Baldantoni *et al.* 2014). Metal hyperaccumulator plants are characterized by having exceptionally high concentrations of metals in their aboveground biomass (Reeves 1992; Brooks 1998; Van der Ent *et al.* 2013). Many studies have defined hyperaccumulators of lead to be species with shoot concentrations of 1000 μg g^{-1} Pb with approximately 0.1 % dry weight (Watanabe 1997; McGrath, Zhao, and Lombi 2001; Reeves 2006; Padmavathiamma and Li 2007). One of the plant accumulating mechanisms is locating the metals by means of the roots, which leads to a root biomass increase in soils enriched with metals, which is known as a positive response (Whiting *et al.* 2000). It is noteworthy that most hyperaccumulator species are endemic to metalliferous soils and behave as "strict metallophytes," while others are "facultative metallophytes" because they can also live in non-metalliferous soils (Cappa and Pilon-Smits 2014). Therefore, in order to implement a phytoremediation program, it is important to select appropriate species for each situation (Baker *et al.* 1994; Gunawardana, Singhal, and Johnson 2011), for which the study of native species as potential accumulators of metals is necessary, considering the risks involved in the introduction of exotic species (Robinson *et al.* 1997; Chaney *et al.* 1999). Further knowledge regarding species selection should be obtained by considering the biological, geochemical and climatic factors that modify

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the bioavailability of metals (de Souza *et al.* 1999; Lin *et al.* 2004; Kuffner *et al.* 2008; Kabata-Pendias 2004). The rhizosphere is where interaction among microorganisms, roots and the soil takes place (Lynch 1990), with studies having reported the release of hydrogen ions, exudates and metabolites into the rhizosphere, which can alter the pH of the soil solution and thus the availability of nutrients such as iron and nitrogen as well as heavy metals in soil (Marschner and Römheld 1983; Lin *et al.* 2004). Microcosm studies using simple, fast and non-destructive techniques have been effective to locate the active root zone, in which it is possible to measure the changes related to the availability of a metal. For example, devices such as the rhizobox have been utilized, which basically consists of PVC boxes equipped with transparent observation windows (Neumann, George, and Plassard 2009), as an effective tool for the visualization of roots.

Nevertheless, it is still necessary to extend the studies related to the mechanisms that take place in the rhizosphere and determine the bioavailability of metals in order to improve the phytoextraction effectiveness of the species used in phytoremediation programs. Therefore, the objective of this study was to analyze and compare the behavior of the root system for the accumulator species as Indian mustard [*Brassica juncea* (L.) Czern.] and a native species of the study area (*Bidens pilosa* L.), when exposed to different lead concentrations in soils, through chemical and visualization techniques of the rhizosphere.

Materials and methods

In our experiments, all the reagents were analytical grade and used without further purification. The reagents NaOCl, KCl, KNO_3, NH_4NO_3, and bromothymol blue and $Pb(NO_3)_2$ were purchased to Merck Millipore (Germany). The Ga standard, NaOAc and $MgCl_2$ were purchased to Sigma Aldrich (Germany).

Collection of plant and soil material

Soil samples employed in the rhizobox systems were sampling in the town of Bouwer in Córdoba Province, Argentina, which is characterized by the presence of a former battery recycling plant (31° 33′34.02″ S; 64° 11′9.05′′ W). At this location agricultural topsoils were collected in 3 areas with different levels of pseudototal lead in soils: low (<10 mg kg^{-1}), medium (~ 300 mg kg^{-1}) and high (~ 1000 mg kg^{-1}), which were situated at different distances from the former Pb smelter following previous studies (Salazar 2013; Salazar and Pignata 2014). Soil sampling procedures were performed according to Rodriguez *et al.* (2014). Briefly, topsoil samples were collected at three sampling sites, with each site consisting of a 25 m^2 square with 9 sub-sampling points, systematically arranged with a 2.5 m gap between them. Topsoil subsamples were collected at the nine points using a blasthole for composite soil samples at a depth of 0–10 cm, with stones and foreign objects being removed by hand. Soil was kept in plastic bags under controlled conditions of temperature and humidity (20°C and 60%, respectively) until being processed in the laboratory.

The seed collection of the native species *Bidens pilosa* L. was carried out during the mature stage (March-April) in the study area, taking into account that this species has been previously mentioned as being a potentially suitable species for phytoremediation of soil contaminated with Pb (Salazar and Pignata 2014). In addition, commercial Indian mustard [*Brassica juncea* (L.) Czern.] seeds (Rareplants, Germany; code BJ 3870) were employed with the purpose of comparing the response to that of the native species. Indian mustard was chosen considering that many studies have reported the ability of this species to accumulate lead in its aerial tissues (Liu *et al.* 2000).

Seedling preparation

B. juncea and *B. pilosa* seeds were surface sterilized for 10 min with 1% NaOCl, rinsed with deionized water, and germinated for 4 or 5 days at 20°C in laboratory on moist soil with concentrations of metals below the permitted levels (CCME, 1991). Seedlings of each species were germinated in moist chambers (85% relative humidity) and placed in the dark for two days. Then, seedlings were put in illuminated conditions (autumn photoperiod) until the main roots reached a length of about 3–4 cm. Subsequently, the main root of each seedling was cut to produce its bifurcation. Thereafter, seedlings were transferred individually to a 0.05 L glass container with a continuously aerated nutrition solution: 5.8 g L^{-1} KCl, 8.5 g L^{-1} KNO_3 and 5.3 g L^{-1} NH_4NO_3.

Rhizobox design and preparation

A rhizobox system was used for the purpose of visualizing the changes that occurred in the rhizosphere, because this system allows the separation of soil layers at a defined distance from the roots (Kim, Owens and Kwon 2010). A rectangular rhizobox design was constructed using square petri dishes (90 mm high × 90 mm wide × 15 mm deep, Senna Model 3-21016) with a 10 mm slot cut into the top to allow the stem to pass through (Fig. 1a). The boxes were filled completely with soil of different concentrations of lead (control, medium and high). In this study, a modified juxtaposed rhizobox design was employed (Whiting *et al.* 2000), with two adjacent rhizoboxes being in contact along one of their sides (Fig. 1b). In this way, the juxtaposed rhizobox design consisted of homogenous or heterogeneous pairs of boxes (Table 1).

A 14-d-old seedling with the first pair of fully expanded leaves and a bifurcated root, for each species, was transferred to the slot of each juxtaposed rhizobox system, with one of the roots entering each box (Fig. 2). The system was immediately irrigated with deionized water. Then, the juxtaposed rhizobox system was individually wrapped in aluminium foil in order to exclude light from the root zone, and laid at an angle of 75° with the shoot at the top and the lid on the underside to ensure root growth at the soil surface and optimal viewing of the root system. Seedlings were grown under controlled conditions (temperature range 8–20°C, relative humidity among 50–70%, and light exposure corresponding to autumn photoperiod) in a greenhouse for 30 d from the Multidisciplinary Institute of Plant Biology (CONICET).

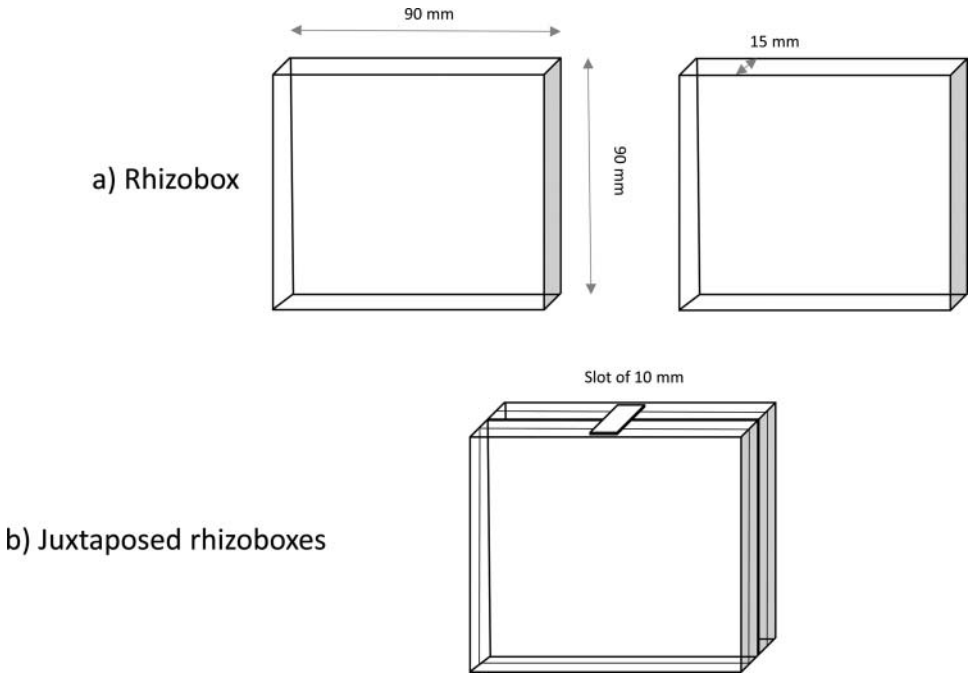

Figure 1. Rhizobox design.

Visualization techniques

Total length and density of roots

In order to assess the root system development as well as the root response under different concentrations of lead in soils, pictures of the roots in the boxes were captured and then analyzed using the programs ImageJ (Rasband 2014) and SmartRoot (Lobet, Pagés, and Draye 2011). A manual tracing of the roots was made, and the total length and density of roots in the rhizobox were calculated.

Analysis of the pH of the root system

This analysis was performed for the purpose of detecting the active root zone, which corresponds to nutrient availability (Dinkelaker *et al.* 1993; Engels *et al.* 2000; Whiting *et al.* 2000; Neumann, George and Plassard 2009), and to assess whether this was affected by the levels of lead in soil. In this study, whole roots were carefully separated from the soil and placed in a petri dish, and then an agarose gel with an acid-base pH indicator (bromothymol blue) was added and the colour changes were visualized according to Häussling *et al.* (1985). An agarose gel blank with the acid-base pH indicator without the application of roots was performed with the purpose of discount the background.

Table 1. Treatments used in the rhizobox systems.

Treatment	Content of Pb in soil
Homogeneous	Control – Control (C-C) Medium – Medium (M-M) High – High (H-H)
Heterogeneous	Control – Medium (C-M) Control – High (C-H) Medium – High (M-H)

Assessment of the lead extraction potential of exudates in the rhizosphere

This experiment was performed in order to evaluate the lead extraction capacity of the rhizosphere exudates. First, 4-d-old seedlings of *B. juncea* and *B. pilosa* were prepared as mentioned in subsection 2.2., which were then transferred to hydroponic conditions for 21-d with Hoagland nutrient solution and with Pb at a concentration of 100 mg L^{-1} added as $Pb(NO_3)_2$, which corresponds to bioavailable Pb concentration of moderate contamination in the study area (Salazar and Pignata 2014). Briefly, hydroponic cultures were performed in opaque plastic containers (4 L) previously washed with 1% HNO_3 and rinsed tree times with deionized water. A styrofoam plate was fitted to each container's mouth to provide physical support to plants,

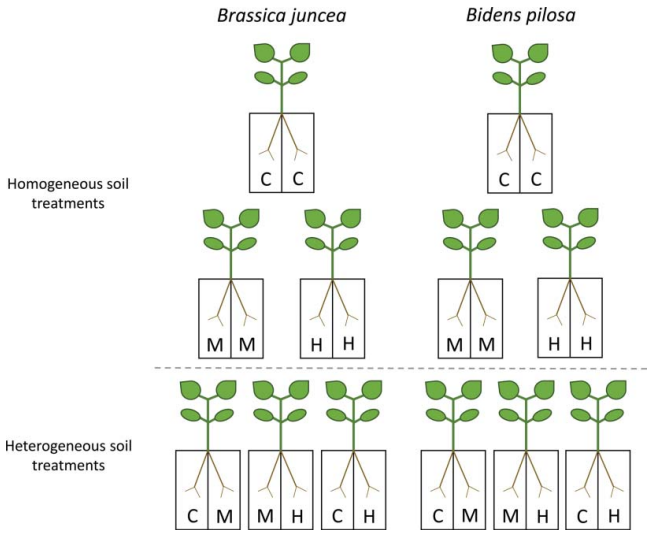

Figure 2. Rhizobox model with forked roots.

as well as to ensure that only roots were in contact with the culture medium. The nutrient solution previously described was used as growth medium with deionized water (1:2), in addition to the $Pb(NO_3)_2$; This solution was continuously aerated employing a commercial air pump. Once the plant-container assembly was prepared, containers were individually wrapped with a black plastic bag to reduce water evaporation and prevent the incidence of light on the hydroponic medium.

Using the same procedure, a control for each species was made, but without the addition of Pb. Triplicate seedlings of each species were maintained under constant aeration, at controlled conditions in a greenhouse (temperature range 8–20°C, relative humidity among 50–70% and corresponding to autumn photoperiod). Subsequently, each seedling for each treatment was transferred to a 50 mL tube with 40 mL of ultrapure water, and kept there for a period of 30 h. In addition, a blank was prepared without the presence of the seedling. Next, the seedlings were removed and pH and electrical conductivity (EC) were determined in 3 mL of the solution using a pH meter (Altronix TPX II) and an EC-meter with a glass electrode (Oakton WD-35610), respectively. Of the remaining solution, 20 mL were mixed with 1 g of soil with a Pb concentration of 1000 mg L^{-1} and agitated at room temperature for 6 h with the aim of assess the Pb extraction power of each exudates sample. Thereafter, tubes were centrifuged and 3 mL of the supernatant was taken for pH and EC determination as described above, while 0.5 mL was separated for Pb analysis by X-ray fluorescence at the National Laboratory of Synchrotron Light (LNLS) in Brazil. For this determination, 10 mg L^{-1} of Ga was added as an internal standard, and then aliquots of 5 μL were taken from this solution and dried on an acrylic support. Standard solutions with known concentrations of different elements were prepared to calibrate the system.

The samples were measured for 200 s, using the total reflection set up mounted at the X-ray fluorescence beamline. For the excitation, a white beam (approximately 0.3 mm wide and 2 mm high) was used. For the X-ray detection, a HPGe detector was used with an energy resolution of 148 eV at 5.9 keV.

As a quality control, blanks were prepared in the same way and run after five determinations to calibrate the instrument. All results were found to be within ± 2% of the certified value. Finally, the coefficient of variation of the replicate analysis was calculated for different determinations, with the variations being found to be less than 10%.

Lead content in plants and soils

Lead content in soils

In the present study, the concentration of lead for each level (control, medium and high), was analyzed before the experiments in order to corroborate the lead concentrations of these soils. A sequential extraction in two stages (mobile and mobilizable) was performed according to Tessier, Cambell and Bisson (1979). In this method, the first fraction indicates the exchangeable fraction of the metal in soil, while the second fraction indicates the carbonate bound fraction. Therefore, the sum of these fractions represents the bioavailable fraction of lead to plants (Gleyzes, Tellier, and Astruc 2002), which was used in this study. Briefly, topsoils were sieved with a stainless steel mesh of 63 μm and then the sequential extraction was performed. The mobile or exchangeable fraction (I) was analyzed and a solution of $MgCl_2$ 1 M (1:8 W/V) was added to the soil samples, which were then agitate for 1 hour and subsequently centrifuged at 350 rpm for half an hour.

Residual soil from the previous step was used to extract the fraction bound to carbonates or the mobilizable fraction (II), by using a suspension of NaOAc 1 M (pH 5) and continuously stirring for 5 hours at room temperature. After the solution was filtered, the lead determinations for the two fractions were analyzed using a Perkin-Elmer AA3110 flame atomic absorption spectrometer (Norwalk, CT, USA). Calibration curves and blanks were performed to assure good quality of the measured data.

Biomass and lead content in B. pilosa and B. juncea

After carrying out the visualization techniques, plants were washed with ultrapure water and separated into roots and aerial parts, which were dried (16 h at 65°C) and weighed (Ohaus AX224/E, Argentine) for biomass determination.

The determination of Pb content in roots and aerial parts was performed using 1 g DW of plant material, which was reduced to ash at 450 °C for 4 h in porcelain crucibles and then digested using 20% HNO_3 for 24 h. The solid residue was separated by centrifugation (Giumelli Z-29-D, Argentine), and the volume adjusted to 5 mL with Milli-Q water (Millipore Simplicity™, Germany). The concentration of Pb was measured using a flame Perkin Elmer AA3110 spectrophotometer.

Quality control

As a quality control, blanks and samples of the standard reference material "CTA-OTL-1" (oriental tobacco leaves, Institute of Nuclear Chemistry and Technology, Poland) for plants, and "BAM-U113" (soil, Federal Institute for Material Research and Technology, Germany) for soils, were prepared in the same way as described above for plants and soils, and were run after ten determinations to calibrate the instrument and monitor the potential sample contamination during analysis. These results were found to be between 87% and 93% of the certified value for CTA-OTL-1 and between 85% and 91% for BAM-U113, with the data error being low and typically less than 15%. The coefficient of variation of the replicate analyses was calculated for different determinations, and variations were found to be less than 10%.

Data analyses

Statistical analyses

The Analysis of Variance (ANOVA) assumptions were previously verified graphically (residual vs. fitted values, box plots, and stem leaf plots). Whenever the ANOVA indicated significant effects ($p < 0.05$), a pairwise comparison of means was undertaken using Fisher's Least Significant Difference (LSD). Analyses were performed using the software Infostat® coupled with R, version 2012.

Translocation and bioconcentration factors

In this study, the translocation factor (TF) (Barman *et al.* 2000; Baker *et al.* 2000) and the bioconcentration factor (BCF) (Baker *et al.* 2000; Komárek, Chrastný, and Štíchová 2007) were calculated using the concentration of Pb in the roots and aerial parts [TF = C aerial / C roots] and the concentration of Pb in soils and aerial parts [BCF = C aerial / C soil], respectively. The TF was calculated in order to analyze the phyto- extractor ability of the species and to identify the accumulation organ, which was obtained by dividing the concentration of lead in the aerial part of the plant by the concentration of this metal in the roots. Values higher than one indicate that the metal was easily translocated, while values below one point to a higher accumulation in roots (Yoon *et al.* 2006).

In addition, in this study, a modified bioconcentration factor (BCF) was calculated by using the ratio of the metal concentration in aerial parts and the "potentially available" metals in the rhizospheric soils, and corresponded to the sum of the mobile and mobilizable fractions of the sequential extraction of metals (Komárek, Chrastný, and Štíchová 2007; Salazar *et al.* 2012; Salazar and Pignata 2014). Thus, as the BCF indicates the ability of plants to accumulate metals, this factor can be used to compare the ability of the different species employed in phytoremediation programs. A BCF <1 indicates exclusor species and a BCF >10 indicates hyperaccumulator species (Yonn *et al.* 2006).

Results and discussion

Lead concentrations in soils

Results of bioavailable lead content in soil showed values of 2.81 ± 0.22 for the control treatment (C), 124.41 ± 30.4 for the medium treatment (M) and 464.06 ± 34.65 for high treatment (H), respectively.

Plant growth in lead enriched soils

Homogeneous soil treatments

The homogeneous treatment was carried out as a control to validate the experiment. Taking into account that concentrations of Pb in the soil of the juxtaposed boxes in the homogeneous treatments were the same, it would be expected to not find significant differences for root length between juxtaposed boxes. Indeed, ANOVA results between boxes of the rhizobox system did not show significant differences in 84% of the cases, from a total of 6 homogeneous treatments for the studied species, thereby confirming the reproducibility of the treatments.

Development of the root system

Table 2 shows the ANOVA results and the mean values ± SE of total length and lead concentration in roots measured for the homogeneous treatments (control-control; medium-medium, and high-high) for *B. juncea* and *B. pilosa*.

Although a decrease of root length with increasing lead concentration in soil was observed, these differences were not significant, indicating tolerance to high concentrations of lead in soil of the studied species.

Table 2. Mean values ± SE and ANOVA results of total length and lead concentration in roots measured in homogeneous treatments (C-C; M-M and H-H) for *B. juncea* and *B. pilosa*.

Species	Treatment	Pb (μg g^{-1} DW)	Total root length (cm)
B. juncea	**C-C**	14.56 ± 8.02 c	292.44 ± 116.10
	M-M	191.35 ± 67.83 b	190.67 ± 82.56
	H-H	1361.69 ± 32.08 a	153.10 ± 49.55
	ANOVA		ns
B. pilosa	**C-C**	28.92 ± 10.91 c	595.06 ± 169.93
	M-M	204.23 ± 37.64 b	534.79 ± 187.40
	H-H	627. 80 ± 144.37 a	526.27 ± 213.82
	ANOVA		ns

Values in each column followed by the same letter do not differ significantly at $p < 0.05$. Significance levels: ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviations: C-C, control-control; M-M, medium-medium; H-H, high-high.

Regarding to Pb accumulated in roots, our results revealed significant difference for both species, with an increase in the concentration of Pb in the roots corresponding to greater concentrations of Pb in the soil in the order H-H> M-M> C-C. These results indicate a direct relationship between the concentration of Pb in soils and its uptake and accumulation in roots of both species, which is in agreement with other studies performed using *B. juncea* (Liu *et al.* 2000; Lin *et al.* 2004; Meyers *et al.* 2008) and *B. pilosa* (Salazar and Pignata 2014).

A comparison between the results for the native species *B. pilosa* and the model species *B. juncea* is shown in Figure 3. The accumulation of Pb in roots revealed significant differences only for the treatment with the highest concentration of lead in soils, with *B. juncea* showing a greater accumulation of Pb in roots (Fig. 3a), in agreement with numerous studies performed on *B. juncea* that reported the ability to accumulate lead (Liu *et al.* 2000; Palmer, Warwick and Keller 2001; Lin *et al.* 2004; Meyers *et al.* 2008). In contrast, *B. pilosa* presented significantly longer roots than *B. juncea* for the M-M and H-H treatments. It is important to note that the root density of *B. pilosa* exposed to high concentrations of Pb was 4.33 cm m^{-2}, which was 3.44 times greater than that for *B. juncea* (Fig. 3b), thus revealing a greater tolerance to high concentrations of lead in soil.

Plant aerial part development

Table 3 shows the ANOVA results and mean values ± SE of biomass and lead concentration measured in aerial parts of *B. juncea* and *B. pilosa* in homogeneous treatments.

A significant increase in Pb concentration in aerial parts with increasing Pb content in soil was observed in both species. However, no significant differences in aboveground biomass among different homogeneous treatments were observed. Regarding lead accumulation in aboveground biomass, other studies have reported similar results for *B. juncea* in which most lead accumulation occurred at the roots with the translocation and accumulation of Pb in aerial parts being directly related to its concentration in the substrate (Liu *et al.* 2000; Zaier *et al.* 2010). However, in contrast with our results, other authors argue that Pb accumulates in *Brassica species* organs in the order leaves> stems> seeds> roots, and have reported values of Pb between 1,416 to 18,812 μg g^{-1} DW (Nanda Kumar, Dushenkov and Raskin 1994). In addition, it is important to note that in the present study the aerial biomass was not affected by the different concentrations of Pb in soils, since no

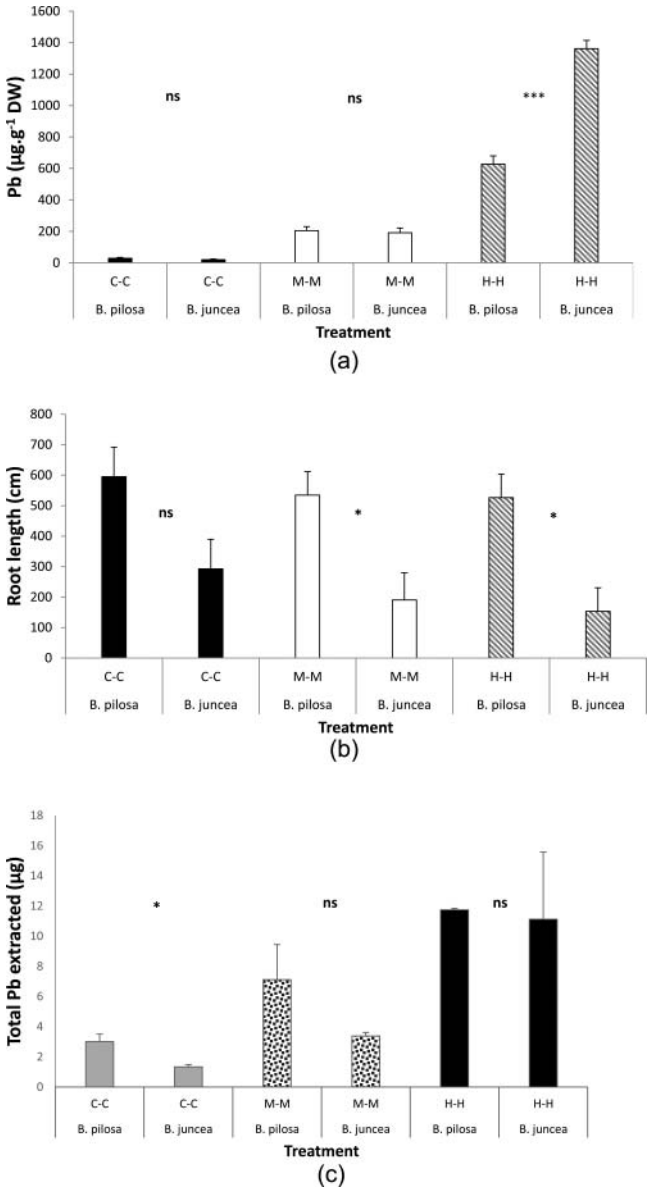

Figure 3. Comparison between *B. pilosa* and *B. juncea*. ANOVA results for Pb content in roots (A), length of roots (B) and total lead extracted (C) of homogeneous treatments (C-C, M-M and H-H). Note: ANOVA: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, not significant.

effective metal translocation was observed to the aerial part in both species, whereas Zaier *et al.* (2010) reported a decrease in aerial dry matter for *B. juncea* at higher lead concentrations.

The Pb values obtained in the present study for aerial biomass in *B. juncea* are much lower than those suggested by other researchers, which we believe was due to the different experimental conditions, since the majority of other studies were performed in hydroponic conditions that do not represent the natural soil conditions. It is important to note that metal availability is affected by physical, chemical and biological factors (Kabata-Pendias and Sadurski 2004).

Regarding the comparison between the species studied, only the H-H treatment revealed a significantly greater accumulation of Pb in the aerial part of *B. juncea* (Fig. 4b). Whereas the comparison of biomass between the species showed a significantly higher value in *B. pilosa* for the M-M and H-H

Table 3. Mean values ± SE and ANOVA results corresponding to Pb content and aerial biomass of *B. juncea* and *B. pilosa* measured in homogeneous treatments (C-C, M-M, and H-H).

Species	Treatment	Pb (μg g^{-1} DW)	Biomass (g DW)
B. juncea	**C-C**	1.97 ± 0.12 c	0.68 ± 0.11
	M-M	6.45 ± 1.13 b	0.53 ± 0.06
	H-H	17.24 ± 1.36 a	0.64 ± 0.21
ANOVA			ns
B. pilosa	**C-C**	3.05 ± 0.47 b	0.99 ± 0.01
	M-M	7.31 ± 2.39 b	0.97 ± 0.001
	H-H	12.01 ± 0.20 a	0.98 ± 0.01
ANOVA			ns

Values in each column followed by the same letter do not differ significantly at $p < 0.05$. Significance levels: ns, not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Abbreviations: C-C, control-control; M-M, medium-medium; H-H, high-high.

treatments (Fig. 4a). However, when the total lead extracted (TE) from the aerial biomass was considered, only significant differences were observed between species for the control treatment, with TE being higher for *B. pilosa* (data not show). Thus these results suggest that, at high concentrations of lead in soil, both species had similar total lead extraction capacities in aboveground parts. These findings are similar to those mentioned above for length and density of roots, where the native species *B. pilosa* showed a greater development than the model species *B. juncea*, thus indicating the ability of the native species to tolerate high concentrations of lead in soil, an essential feature of a phytoextractor species used in phytoremediation programs.

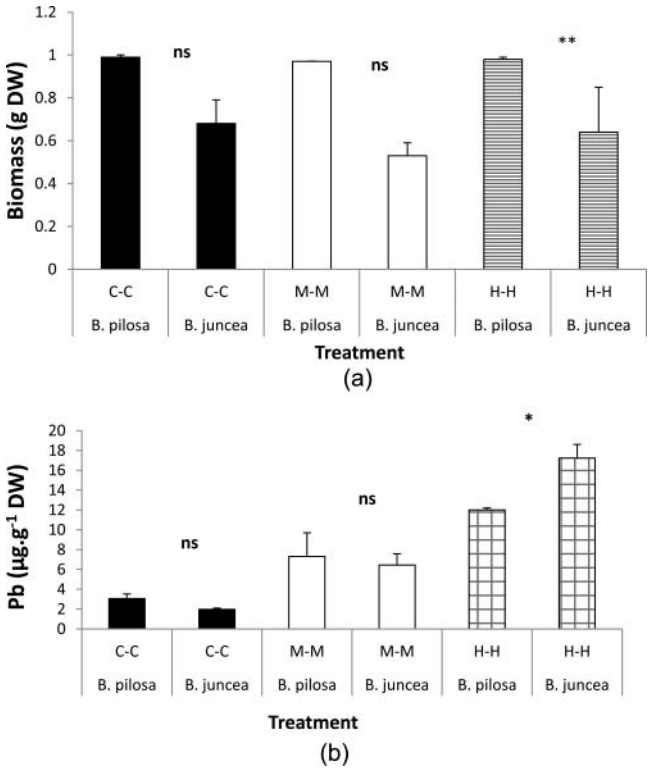

Figure 4. Comparison between *B. pilosa* and *B. juncea*. ANOVA results for aerial biomass (A) and Pb content (B) of homogeneous treatments (C-C, M-M and H-H). Note: ANOVA: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, not significant.

Table 4. Lead concentration ($\mu g\ g^{-1}$) in roots and aerial biomass, translocation factor (TF) and bioconcentration factor (BCF) measured in *B. juncea* and *B. pilosa* for different homogeneous treatments.

	Brassica juncea			*Bidens pilosa*		
Organ	C-C	M-M	H-H	C-C	M-M	H-H
Aerial	1.97 ± 0.12 c	6.45 ± 1.13 b	17.24 ± 1.36 a	3.05 ± 0.47 b	7.31 ± 2.39 b	12.01 ± 0.20 a
Roots	14.56 ± 19.75 c	191.35 ± 22.8 b	1361.69 ± 19.75 a	28.92 ± 43.18 c	204.23 ± 43.18 b	627. 80 ± 144.37 a
TF	*0.14*	*0.03*	*0.01*	*0.11*	*0.04*	*0.02*
BCF	*1.07*	*0.03*	*0.02*	*1.62*	*0.03*	*0.001*

Values in each row followed by the same letter do not differ significantly at $p < 0.05$. Significance levels: ns, not significant, $^{*}p < 0.05$,$^{**}p < 0.01$,$^{***}p < 0.001$. Abbreviations: M, medium; H, high.

Translocation and bioaccumulation of lead

The translocation factor (TF) and bioconcentration factor (BCF) values for different concentrations of Pb in soil in *B. juncea* and *B. pilosa* are shown in Table 4. According to the category usually used for BCF and TF, the results of this study suggest that these species do not behave as hyperaccumulators. It is also important to note that in both species TF decreased with increasing concentration of Pb in soil, which is consistent with the values reported by Salazar and Pignata (2014). Therefore, these results suggest that none of the studied species meet the criteria for being hyperaccumulators, since they have a tendency to accumulate more Pb in the roots than in the leaves and stems. However, it is important to note that for Pb the condition of hyperaccumulator is very difficult to achieve for most plant species, since the roots are the main impediment to translocation to the aerial part (Blaylock and Huang 2000). Meyers *et al.* (2008) have reported that *B. juncea* has an efficient cell root vacuolar storage mechanism in the root tips, which could be regarded as a defense and adaptation strategy to elevated levels of Pb in the root cells.

A higher translocation of lead (TF) was revealed for *B. juncea* in the control treatment compared to the native species *B. pilosa*, but a lower one for the treatments of high and medium concentration of Pb in soils, where *B. pilosa* was revealed to be a better translocator. This feature is very important, since it is a measure of the ability of a plant to transport metal from the roots to the upper portion, where it then accumulates. In addition, the bioconcentration factor showed the same trend as TF, which decreased with increasing concentration of Pb in the soil. The native species *B. pilosa* showed a higher ability to capture lead from soil at low and medium concentrations. However, at the high concentration of Pb in soil, these values were lower in comparison to those of *B. juncea*.

Table 5. Mean values ± SE and ANOVA results of total length and lead concentration in roots measured in heterogeneous treatments (C-M and M-C) for *B. juncea* and *B. pilosa*.

Species	Root	Pb ($\mu g\ g^{-1}$ DW)	Total root length (cm)
Brassica juncea	C	42.41 ± 23.97	156.8 ± 87.12
	M	160.98 ± 33.94	269.66 ± 103.17
ANOVA		ns	ns
Bidens pilosa	C	90.7 ± 52.82	648.92 ± 159.67
	M	158.57 ± 58.00	413.72 ± 90.55
ANOVA		ns	ns

Values in each column followed by the same letter do not differ significantly at $p < 0.05$. Significance levels: ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviations: C, control; M, medium.

Table 6. Mean values ± SE and ANOVA results of total length and lead concentration in roots measured in heterogeneous treatments (C-H and H-C) for *B. juncea* and *B. pilosa*.

Species	Root	Pb ($\mu g\ g^{-1}$ DW)	Total root length (cm)
Brassica juncea	C	35.55 ± 13.72 b	152.56 ± 50.12
	H	860.95 ± 110.82 a	203.46 ± 41.03
ANOVA			ns
Bidens pilosa	C	37.79 ± 24.16 b	371.04 ± 45.81 b
	H	630.74 ± 24.26 a	1010.22 ± 319.47 a
ANOVA			

Values in each column followed by the same letter do not differ significantly at $p < 0.05$. Significance levels: ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviations: C, control; H, high.

Heterogeneous soil treatments

Heterogeneous treatments had different concentrations of Pb in soil in each of the juxtaposed boxes of the rhizobox (C-M, C-H, and M-H), which were performed in order to evaluate the behavior of a plant when it has two different concentrations of lead available in soils by utilizing a forked root design. For each of these treatments (boxes), both sides of the bifurcated root were analyzed.

Development of the root system

Control-medium treatment

Table 5 shows the ANOVA results and mean values ± SE of total length and lead concentration in roots measured in the heterogeneous treatment Control-Medium (C-M) for *B. juncea* and *B. pilosa*.

Table 7. Mean values ± SE and ANOVA results of total length and lead concentration in roots measured in heterogeneous treatments (M-H and H-M) for *B. juncea* and *B. pilosa*.

Species	Root	Pb ($\mu g\ g^{-1}$ DW)	Total root length (cm)
Brassica juncea	M	174.09 ± 57.83 **b**	169.7 ± 80.33
	H	1652.46 ± 229.44 **a**	119.88 ± 96.34
ANOVA			ns
Bidens pilosa	M	234.63 ± 116.50	526.89 ± 17.55
	H	414.32 ± 32.00	475.48 ± 88.15
ANOVA		ns	ns

Values in each column followed by the same letter do not differ significantly at $p < 0.05$. Significance levels: ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Abbreviations: M, medium; H, high.

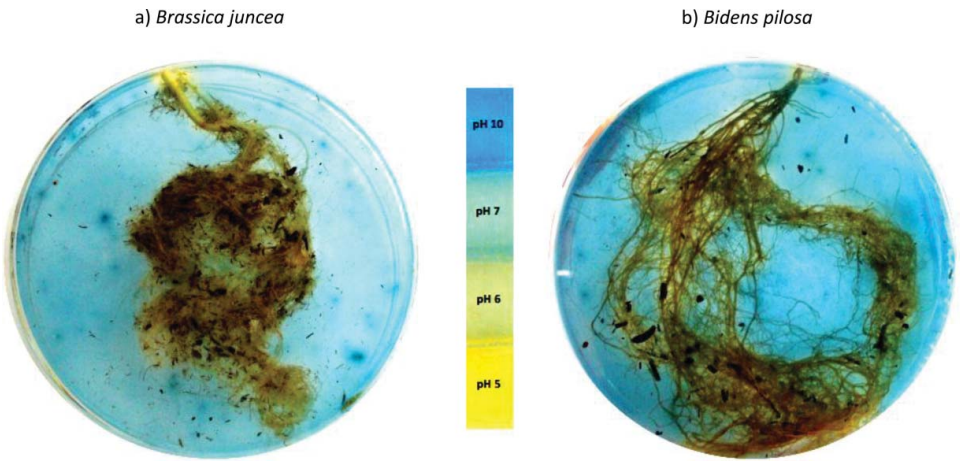

Figure 5. Visualization of root-induced pH changes in the rhizosphere using indicator media applied to the root surface of plants grown in rhizobox systems for *B. juncea* (A) and *B. pilosa* (B).

Although a trend of increased accumulation of lead in the roots corresponding to soils of Medium treatment (124.41 ± 30.4 mg.kg^{-1} Pb bioavailable in soils) for both species was observed, this difference was not significant. Regarding the length of the roots, no significant differences were observed for any of the species tested.

Control-high treatment

Table 6 shows the ANOVA results and mean values ± SE of total length and lead concentration in roots measured in the heterogeneous treatments Control-High (C-H) for *B. juncea* and *B. pilosa*.

It was observed that Pb content and the length of roots were significantly greater in the treatment corresponding to the high concentration of Pb in soil. Thus, these results revealed a positive response of the root system to high concentrations of Pb in soil for both species.

Medium-high treatment

Table 7 shows the ANOVA results and mean values ± SE of total length and lead concentration in roots measured in the heterogeneous treatments Medium-High (M-H) for *B. juncea* and *B. pilosa*.

The results showed a significant increase of lead in the roots of *B. juncea* for this treatment of a higher concentration of lead in soil. A similar behavior was also observed for *B. pilosa*. However, these results were not significant, which suggests that the model species *B. juncea* accumulates a larger amount of Pb when the metal is available in the soil, unlike the native species. Furthermore, with respect to the root length, no significant differences between treatments were observed, indicating a high tolerance to lead. It is important to note that in all cases, the root density of *B. pilosa* achieved higher values than *B. juncea* (data not shown), although the latter accumulated more lead.

Table 8. Mean values ± SE and ANOVA results corresponding to lead extractive potential of exudates in the rhizosphere of *B. juncea* and *B. pilosa*.

Species	Treatment	Pb (μg g^{-1} DW)	EC (μS cm^{-2})	pH
B. juncea	Development without Pb	8.55 ± 5.09 b	73.90 ± 4.10	5.59 ± 0.02
	Development with Pb	25.61 ± 5.00 a	64.20 ± 5.02	5.53 ± 0.03
ANOVA			ns	ns
B. pilosa	Development without Pb	35.57 ± 4.07 a	68.53 ± 5.04	5.59 ± 0.10
	Development with Pb	22.88 ± 3.57 b	74.57 ± 4.24	5.58 ± 0.09
ANOVA			ns	ns

Values in each column followed by the same letter do not differ significantly at $p < 0.05$. Significance levels: ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Effect of rhizosphere exudates on pH

Regarding the staining homogeneity, these results generally revealed a homogeneous staining for *B. pilosa*, while in contrast for *B. juncea*, the basal parts of the main roots were the most stained, and the rhizosphere of the secondary and root apex were the least stained (Fig. 5), which is consistent with other studies reporting differences in pH with a reduced capacity of the rhizosphere between the lateral and main roots for *A. murale* and *R. sativus* (Kidd *et al.* 2009).

No pattern was revealed between the different treatments and the changes in pH within species. B. juncea showed, in general, a rhizosphere pH range of 5–6, whereas the rhizosphere of *B. pilosa* had a slightly acidic value of 5. Therefore, these results indicate that the rhizosphere pH did not respond to changes in the concentration of lead in the soil. In contrast, other studies have reported that plants have the ability to transform metal fractions for easier uptake through root exudation or pH changes in the rhizosphere (Hinsinger *et al.* 1993; Wang *et al.* 2009; Li *et al.* 2014). However, most of these studies did not consider the buffer soil capacity, which varies between different soils types, and which therefore may be the main factor involved in the solubilization of metals in soils (Knight *et al.* 1997; Luo, Christie, and Baker 2000).

Lead extraction potential of exudates in the rhizosphere

Table 8 shows the ANOVA results and mean values ± SE of the lead extraction potential of exudates in the rhizosphere of *B. juncea* and *B. pilosa*. It can be noted that the exudates of both studied species revealed different potentials of lead extraction in relation to the conditions in which they grew. In this way, the model species *B. juncea* showed an increase in its ability to extract lead when seedlings were grown in a Pb enriched environment, whereas the opposite behavior was observed for the native species *B. pilosa*, whose exudates extracted more lead when the seedlings were grown in a Pb-free environment. This is a highly significant result, since it allows the optimal conditions to be established for growing plants that can later be used for phytoremediation. The results of the physical

parameters, pH and EC of the exudates, did not differ significantly between treatments, with no significant association with the contents of Pb extracted by exudates being observed for the species analyzed.

Conclusions

Moreover, the root system development indicated a positive response in the presence of lead for both studied species, suggesting a tolerance to high concentrations of lead in soil. In addition, the aerial biomass was not affected by the accumulation of lead, revealing another tolerance indicator for both species. The comparison between species indicated that the model species *B. juncea* had a greater ability to accumulate lead in its aerial biomass than the native species *B. pilosa*, while the latter presented a higher root development in the presence of elevated concentrations of lead in soil. The analyzed species were tolerant to high concentrations of lead in soil, and accumulated the metal mainly in their roots and to a lesser extent in the aerial compartments, with these concentrations being lower than those quoted in other studies performed with the model species *B. juncea*. This difference in lead concentrations in plants was very likely due to the varying availabilities of the metal in different experimental conditions, as most of the other studies were performed in hydroponic conditions (rather than in naturally lead enriched soils as in the case of the present study), where the incorporation and subsequent accumulation of lead in the plant is impaired. Different responses of potential extraction of lead for exudates were found between species, which is an important finding taking into account the growth conditions of the species used in phytoremediation. It is noteworthy that the native species *B. pilosa* presented tolerance to high concentrations of lead in soil, showing a positive tropism of the root system to lead and has the advantage of being an annual species, which are important characteristics to be employed in phytoremediation programs.

In conclusion, it is important to note that although the species assessed in this study did not show high extraction rates of lead, they exhibited root system properties that clearly favored the uptake and translocation of lead. Thus, the efficiency of the mechanisms involved in these processes should now be enhanced, e.g., through genetic transformation, in order to be able to use them in future phytoremediation programs.

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