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Auxin effects on Pb phytoextraction from polluted soils by *Tegetes minuta* L. and *Bidens pilosa* L.: Extractive power of their root exudates

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HIGHLIGHTS

- IAA does not reverse toxicity effects or enhance Pb uptake by *Tagetes minuta* or *Bidens pilosa*.
- *T. minuta* and *B. pilosa* root exudates have a higher Pb extraction power in response to Pb exposition.
- Root exudate Pb extraction power is related to Pb uptake by roots and translocation to stem.
- Pb soil remediation efficiency of *T. minuta* and *B. pilosa* is within the range of other hyperaccumulators.

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ABSTRACT

The principal impediment for Pb uptake by plants is the Casparian strip in roots. It prevents metals reaching the xylem, thereby hampering translocation to the aerial organs. In the root apices, young root cells have thin cell walls and the Casparian strip is not completely developed, which could facilitate Pb uptake by roots at these vulnerable points. However, as the phytotoxic effects of Pb reduce root growth and enhance suberization, entry of Pb into the plant is avoided. We propose that the application of root growth promotors could be an important complement in the phytoextraction of Pb from polluted soils, due to their effects on produced biomass, Pb toxicity, and root exudate production.

A greenhouse experiment was carried on to evaluate the auxin application effect on the Pb uptake of *Bidens pilosa* and *Tagetes minuta*. These species were sensitive to auxins, but the phytotoxic effect of Pb was not reversed by this treatment. Root exudates capable of extracting Pb were produced only when the species were grown in highly polluted soils, indicating a behavioral response to Pb exposure which is desirable for phytoremediation.

1. Introduction

Heavy metal soil pollution is currently considered to be one of the most serious environmental problems due to its persistence and toxicity, which is continuously increasing due to human activities [1]. This environmental problem has a great impact on affected societies, as the development of areas with poor quality soil is difficult and implies carrying out complex and costly measures in order to improve the soil [2]. In fact, the remediation of heavy metal polluted soils represents a technological challenge for both industries and government institutions, with phytoremediation being an alternative that contemplates soil conservation by harnessing the potential of plants to transform or eliminate the contaminants accumulating in their tissues [3]. However, the phytoremediation of polluted soils in the case of Pb is a goal that has still not been achieved, despite considerable scientific research on this issue, with the bibliography pointing out the great difficulty for this pollutant to be translocated to the aerial organs in most plant species, which is the main obstacle to overcome before this practice can be implemented [4,5]. In this context, the most important biological barrier for Pb uptake is the Casparian strip, which is located in the root

Abbreviations: EC, electrical conductivity; OM%, organic matter percentage; C:N:, proportion carbon to nitrogen; DW, dry Weight; TF, translocation factor; R-A:, root to aerial; TE, total extraction; IAA, indolic acetic acid.

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[5,6]. This metal can obtain access to the radical cortex, but not to the central cylinder. Thus, since it does not reach the xylem, translocation to aerial organs is impeded. In root apices, however, young root cells have thin cell walls and the Casparian strip is not completely developed due to the occurrence of an elongation process in this area, which facilitates Pb uptake by roots [7]. Nevertheless, the phytotoxic effects of Pb include the reduction of root growth and the enhancement of root suberization, thereby tending to prevent the entry of Pb into the plant through this vulnerable point [8].

Liphadzi et al. [9] suggested the application of methodologies that can cause a greater root growth, with the aim of improving phytoremediation results for heavy metal polluted soils, and demonstrated that auxin application enhanced Pb extraction from polluted soils by *Helianthus annuus*. Nevertheless, this is not a technique that is necessarily successful in all species. In addition, although Barbafieri and Tassi [10] reported that assisted phytoremediation by plant growth regulators is harmless to the environment, practical and economically viable, only recently have some studies revealed the possibility of applying phytohormones for this purpose. In nature, phytohormones appear in the rhizosphere because microorganisms produce and release them in the context of a positive interaction with plants. These plants produce root exudates with organic compounds that act as nutrients for microorganisms, which liberate hormones to promote root growth and obtain more root exudates [11]. Thus, a relationship between root exudates and presence of phytohormones is well established, in which they are mutually dependent. Root exudates have also been reported due to their important role in solubilizing metals in soil, thereby favoring their uptake by roots [12].

Bidens pilosa L. and *Tagetes minuta* L. are two Asteracea species with a known potential capability for phytoremediation of Pb polluted soils [13]. Both these species are annual and herbaceous plants, that can grow from 0.4 m to 2 m in height depending on the environmental conditions. Although, these species have shown a high tolerance when Pb is present in their tissues, the Pb tends to remain in the roots. Thus, it would be of great interest to be able to achieve a higher Pb extraction rate and translocation factor for these tolerant species.

In the present investigation, we hypothesized that indole-3-acetic acid (IAA), an auxin phytohormone promotor of plant growth, has the capability of enhancing the Pb extraction efficiency of *B. pilosa* and *T. minuta* from polluted soils by producing more biomass or by reducing the Pb phytotoxic effects, thereby permitting higher Pb concentrations in their organs. In considering the relation between phytohormones and root exudates, we also studied the Pb extraction power of the latter.

2. Experimental

2.1. Soil

Superficial soil was collected from the vicinity of a former battery recycling plant in the town of Bouwer, Cordoba Province, Argentina. Three areas with different Pb concentrations in soils were chosen and defined according to previous studies [13,14] as: Low (24.72 ± 3.73 mg Pb kg^{-1}); Medium (295.5 ± 35.5 mg Pb kg^{-1}) and High (1222.63 ± 255.59 mg Pb kg^{-1}), with their physicochemical properties presented in Table 1. Soils were collected at a depth of 0–15 cm using a stainless steel shovel, and foreign objects were removed. They were then placed in plastic bags, and once in the laboratory, they were sieved to $\leq$2 mm (using a polyethylene sieve) and homogenized, with soil from each area being separated into two equal parts to obtain six treatments: (i) High; (ii) High with auxins; (iii) Medium; (iv) Medium with auxins; (v) Low; (vi) Low with auxins.

2.2. Plant materials

Seed collection of the native species *B. pilosa* L. and *T. minuta* L. was carried out during their mature stage (March-April), in the area previously denominated "High". Seeds were sterilized for 10 min with 1% NaOCl, rinsed with deionized water, and germinated for 4 or 5 days at 25 °C in sterilized sand. Seedlings of each species were grown in pots corresponding to the different treatments under controlled conditions (temperature range 18–35 °C; relative humidity between 60 and 70%, corresponding to the summer photoperiod) in a greenhouse for the full growth period (12 weeks).

2.3. Experimental design

Plants were assigned to each one of the 6 treatments in a completely randomized experimental design. They were transplanted to 15 L rectangular pots with a large surface area to simulate field allotment conditions (0.4 m long $\times$ 0.25 m width $\times$ 0.15 m depth), with a total of 24 plants being grown together in each pot, thereby sharing the rhizosphere as occurs in the field. Pots were watered with 3 L of tap water once before plants being transplanted. During the first month pots were watered three times a week with 0.5 L, after which, the larger plant size implied that more water was required, so the pots were watered four times a week with 0.5 L. This watering produced moist but not soaked soils, thereby avoiding anaerobic conditions for the roots since there were no drainage holes in the pots with the aim of preventing Pb or auxin leaching. Auxin indol-3-acetic acid (IAA) was applied to soils and leaves following Liphadzi et al. [9] for the auxin treatments. Three applications were carried out during the sixth, eighth, and tenth week. Then, during the twelfth week, the plants were harvested in two stages; the first one to collect root exudates, and the second to obtain plant samples for Pb analyses. For the first stage, 6 complete plants were carefully removed from each pot, and their roots were washed 3 times with tap water and then 3 times with milli-Q water. Each plant was placed individually in a 50 mL plastic tube, with 30 mL of milli-Q water covering the root system. After 24 h, these plants were collected, and the liquid face was recovered and adjusted to 30 mL with milli-Q water, with this solution being conserved as the root exudate sample [15] for further analyses. For the harvesting second stage, the 6 plants used for the exudate collection and the other 18 remaining in the pot were grouped into 6 pool samples made up of 4 plants each, where each pool contained one plant previously used for the exudate collection and three more plants from the pot. Samples were separated into root, stem and leaves, before being washed and sonicated with milli-Q water and oven dried at 60 °C until constant weight. The dried biomass per plant was determined, and then the samples were ground, homogenized, and conserved for further analyses.

2.4. Determination of Pb in plant organs

The concentration of Pb in plant organs (root, stem and leaves) of *T. minuta* and *B. pilosa* was determined according to Wannaz et al. [16]. To carry this out, 1 g of dried samples was prepared for analysis by total reflection X-ray fluorescence (TXRF) using synchrotron radiation. The plant material was reduced to ash in a muffle furnace at 450 °C for 4 h, and the ash was digested with 3 mL of analytical grade HNO_3 at 25 ± 2C. The solid residue was removed by centrifugation, and the supernatant was filtered with Munktell filter paper of 2 μm (Bärenstein, Germany) and then adjusted to 5 mL with Milli-Q water. Samples were added with a solution of Ga 10 ppm as the internal standard. Aliquots of 5 μL of this solution were pipetted in the center of an acrylic support and standard solutions with known concentrations of Pb were prepared to calibrate the system. The samples were measured for 200 s, using the total reflection set

Table 1
Mean values ± SD and results of the analysis of variance (ANOVA) for physico-chemical properties of soils.

	Soil sample			ANOVA
Parameter	Low	Medium	High	
Distance to the smelter (m)	1011	141	31	
pH	6.53 ± 0.07	6.54 ± 0.07	6.83 ± 0.08	ns
EC ($\mu s\,cm^{-2}$)	61.4 ± 3.91	70.77 ± 3.64	76.54 ± 6.43	ns
OM%	8.91 ± 0.76	9.5 ± 1.3	8.08 ± 0.16	ns
C:N (%)	9.70 ± 0.08	10.11 ± 0.09	9.30 ± 0.10	ns
Particle size distribution (%)				
Sand (2 – 0.05 mm)	16.04 ± 0.35	16.88 ± 1.23	15.91 ± 0.93	ns
Silt (0.05 – 0.002 mm)	82.01 ± 1.13a	82.46 ± 1.48a	78.02 ± 0.14b	***
Clay (<0.002 mm)	1.96 ± 0.78b	0.66 ± 0.25c	6.08 ± 0.78a	***
Pb_I ($mg\,kg^{-1}$)	2.81 ± 0.22c	124.41 ± 30.4b	464.06 ± 34.65a	***
Pb_{II} ($mg\,kg^{-1}$)	24.72 ± 3.73c	295.5 ± 35.5b	1222.63 ± 255.59a	**
Mn_I ($mg\,kg^{-1}$)	64.03 ± 6.50	120.69 ± 14.26	92.87 ± 16.84	ns
Mn_{II} ($mg\,kg^{-1}$)	392 ± 47.04	516.21 ± 14.01	389.88 ± 44.27	ns
Fe_I ($mg\,kg^{-1}$)	112.53 ± 12.76	137.69 ± 18.63	148.78 ± 22.59	ns
Fe_{II} ($mg\,kg^{-1}$)	12495 ± 4721	9276 ± 433.0	13708 ± 5960	ns
Cu_I ($mg\,kg^{-1}$)	6.17 ± 0.48a	4.22 ± 0.18b	6.67 ± 0.435a	*
Cu_{II} ($mg\,kg^{-1}$)	8.6 ± 1.35	9.95 ± 0.19	7.61 ± 0.41	ns
Zn_I ($mg\,kg^{-1}$)	12.43 ± 0.46a	10.81 ± 0.33b	14.39 ± 0.72a	*
Zn_{II} ($mg\,kg^{-1}$)	24.51 ± 5.31	28.56 ± 1.05	29.66 ± 6.81	ns

Abbreviations: $Metal_I$: bioavailable metal (extracted with 1 M $MgCl_2$ pH 7); $Metal_{II}$: total metal concentration (pure HNO_3 extraction). Values in each row (ANOVA) followed by the same letter do not differ significantly at $p < 0.05$. (ns, not significant.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

up at the X-ray fluorescence beamline at the National Synchrotron Light Laboratory (LNLS), Campinas, SP, Brazil, with a white beam (approximately 0.3 mm wide and 2 mm high) being used for excitation. For X-ray detection, a Ge detector was used with an energy resolution of 148 eV, at 5.9 keV, with a 0.8 mm collimator in the detector.

2.5. Exudate extraction power

The collected root exudate samples were used as the extraction solution for a Pb polluted soil. For this purpose, 15 mL of root exudates were mixed with 1 g of soil (the same soil used as in the High concentration treatment, with its physico-chemical properties being presented in Table 1) and stirred for 6 h (as was described for the extraction of the available fraction of metals [17]), before being centrifuged and the supernatant filtered and stored for measurement of metals by TXRF following the same methodology reported for plants. Results were expressed in two ways: (i) concentration (μg of Pb in 1 mL of the extraction solution); and (ii) extraction (total amount of Pb (μg) extracted from 1 g of soil using 15 mL of the extraction solution).

To determine the extraction power of the milli-Q water used to obtain the root exudates, control-blank solutions were prepared by adding 30 mL of milli-Q water to 50 mL falcon tubes, and after 24 h, these were also used as the extraction solution for the same soil.

2.6. 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, Warsaw, Poland) were prepared in the same way as described above for plants, which were run after ten determinations to calibrate the instrument and to check potential sample contamination during analysis. These results were found to be between 83% and 86% of the certified value, with the data errors being low and typically less than 10%. The coefficients of variation of the replicate analyses were calculated for different determinations and found to be less than 10%.

2.7. Statistical analysis

The Shapiro-Wilks test was performed to evaluate the normal distribution of variables, with no data having a non-normal distribution being identified. However, heteroscedasticity was found, which was resolved by its incorporation into the model using Infostat/E coupled to R [18] for the analysis of variance (ANOVA). The experimental design included three factors (species, auxin treatment and Pb concentration in soil), so a multifactorial ANOVA in cells was performed crossing the factor categories, with the aim of enhancing the statistical power and reducing type II errors. Whenever the ANOVA indicated significant effects ($p < 0.05$), a mean pairs comparison was carried out by using the Tukey test.

2.8. Translocation factor and total extraction

The translocation factor from the roots to the aerial parts (TF_{R-A}) was calculated using the ratio between the Pb concentration in the aerial part and that of the root [19]:

$TF_{R-A} = [Pb]_{aerial}/[Pb]_{root}$, with the Pb concentration in the aerial parts being calculated as follows:

$[Pb]_{aerial} = ([Pb]_{leaves} \times Bm_{leaves} + [Pb]_{stem} \times Bm_{stem})/(Bm_{leaves} + Bm_{stem})$

The total extraction (TE) of Pb per plant in each organ was obtained in the following way:

$TE_{leaves} = [Pb]_{leaves} \times Bm_{leaves}$

$TE_{stem} = [Pb]_{stem} \times Bm_{stem}$

$TE_{root} = [Pb]_{root} \times Bm_{root}$

$TE_{plant} = TE_{leaves} + TE_{stem} + TE_{root}$

where [Pb] is the concentration of Pb in the organ indicated in the subscript, and Bm is the total dry biomass produced by each plant in the organ indicated in the subscript.

2.9. Lead extraction efficiency

Lead extraction efficiency is a theoretical variable calculated to estimate the amount of soil (expressed in grams) which one plant is able to clean, by assessing the performance of the phytoremediator. This variable considers the absolute amount of Pb extracted by one plant and the concentration of the pollutant in the soil where the plant is growing. Mathematically, Pb extraction efficiency is the

Table 2
Mean values ± SD and results of the analysis of variance (ANOVA) comparing biomass and Pb concentration in leaves, stem and roots, and translocation factor (TF) from roots to aerial part for *Tagetes minuta* L. and *Bidens pilosa* L. grown in soils with different Pb concentrations and auxin treatment.

Species	Treatment	Soil	Biomass (g DW)			Pb concentration (μg g^{-1} DW)			TF
	ANOVA		Leaves ***	Stem ***	Root ***	Leaves ns	Stem ***	Root ***	R-A *
Tagetes minuta L.	Auxin	Low	1.15 ± 0.06 a	1.68 ± 0.05 a	0.23 ± 0.02 a	3.97 ± 5.14	5.41 ± 5.09 b	7.9 ± 7.6 d	0.12 ± 0.05 b
		Medium	0.39 ± 0.04 c	0.68 ± 0.02 d	0.11 ± 0.01 b	2.85 ± 4.93	1.81 ± 2.01 b	43.2 ± 12.4 c	0.05 ± 0.01 b
		High	0.38 ± 0.07 c	0.51 ± 0.10 d	0.07 ± 0.01 c	3.29 ± 3.17	61.5 ± 12.4 a	741 ± 263 a	0.05 ± 0.02 b
	Control	Low	0.71 ± 0.09 b	1.19 ± 0.21 a	0.19 ± 0.03 b	8.35 ± 7.89	6.25 ± 2.74 b	3.89 ± 3.67 d	1.41 ± 0.9 a
		Medium	0.56 ± 0.07 b	0.96 ± 0.10 d	0.14 ± 0.01 b	6.52 ± 6.14	14.4 ± 20.1 b	50 ± 9 c	0.27 ± 0.13 b
		High	0.44 ± 0.08 c	0.58 ± 0.09 d	0.08 ± 0.01 c	8.24 ± 0.94	54 ± 12 a	646 ± 177 a	0.06 ± 0.02 b
Bidens pilosa L.	Auxin	Low	0.62 ± 0.16 b	2.05 ± 0.71 a	0.28 ± 0.10 a	5.24 ± 3.37	nd	4.43 ± 3.27 d	–
		Medium	0.29 ± 0.10 c	0.55 ± 0.18 d	0.15 ± 0.07 b	5.74 ± 2.21	11 ± 6 b	91 ± 17 b	0.10 ± 0.04 b
		High	0.28 ± 0.17 c	0.59 ± 0.37 d	0.14 ± 0.03 b	15 ± 5	65 ± 13a	1147 ± 188 a	0.10 ± 0.04 b
	Control	Low	0.41 ± 0.04 c	1.00 ± 0.12 c	0.18 ± 0.01 b	2.28 ± 1.95	8.95 ± 8.79 b	4.63 ± 2.17 d	0.66 ± 0.36 a
		Medium	0.35 ± 0.01 c	0.66 ± 0.03 d	0.17 ± 0.02 b	13.4 ± 4.7	5.33 ± 4.66 b	80 ± 21 b	0.04 ± 0.01 b
		High	0.24 ± 0.02 c	0.49 ± 0.09 d	0.09 ± 0.01 c	18 ± 7	59 ± 13a	950 ± 192 a	0.05 ± 0.02 b

Values in each column followed by the same letter do not differ significantly at $p < 0.05$. (ns, not significant). nd: not detectable.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

quotient between the total extraction per plant and the amount of Pb that needs to be extracted from 1 g of soil to attain a 25 μg g^{-1} concentration. Thus,

$$E_{Pb} = TE_{plant}/([Pb]_{soil} - 25\ \mu g\ g^{-1})$$

where $[Pb]_{soil}$ is the concentration of Pb in soil and TE_{plant} is the total extraction of Pb per plant.

3. Results and discussion

3.1. Auxin effect

The auxin and soil Pb concentration effects on *T. minuta* and *B. pilosa* biomass production, on the Pb concentration in leaves, stem and root, and on translocation factor are shown in Table 2. As expected, an enhancement effect of auxin application on plant growth was observed, mainly when plants grew in soils with a low Pb concentration. *T. minuta* leaves and roots, and the whole plant of *B. pilosa* presented significantly more biomass due to auxin application in the absence of Pb pollution. Medium and high Pb concentrations in soils reduced the plant biomass in the three organs, with auxin application being unable to reverse this toxic effect, except for *B. pilosa* root biomass at high Pb soil concentration. With respect to the Pb concentration in plant organs, neither auxin nor Pb concentration in soil had any effects on the Pb concentration in leaves, while the Pb concentration in stem and root was mainly influenced by the Pb concentration in the soil. Both species localized Pb mainly in the root, thus TF R-A presented poor values. The translocation factor revealed values greater than unity only for *T. minuta* in the absence of Pb in soils. However, when auxin was applied, the TF value was reduced.

According to the bibliography, an enhancement of biomass and Pb extraction could be expected due to IAA [9,11,20,21]. Nevertheless, the opposite effect has also been reported, as in results obtained by Kuffner et al. [22], who studied the release of exudates produced by rhizosphere microorganisms and their relationship with the availability of heavy metals in soil. These authors found three bacterial species that produce IAA (the same hormone used in the present study), and for all three cases, the availability of heavy metals was reduced compared to control treatments. In another study, evidence was found of an IAA inhibitory effect on heavy metal incorporation, including Pb, by alga *Chlorella vulgaris* [23]. Analyzing these contradictory findings, it is remarkable that authors who observed an enhancement of heavy metal extraction due to auxin application, in fact worked with soils that presented relatively low pollution levels (total Pb concentration of 139 mg kg^{-1} [9]; total Pb concentration of 150.1 mg kg^{-1} [20]; and soil artificially contaminated with Ni (275 mg kg^{-1}), Cu (300 mg kg^{-1}) and Zn (400 mg kg^{-1}) [21]), values lower than those of our investigation. In these other studies, a growth inhibitory effect of the toxic metals was not found in the cases evaluated; whereas, in our investigation a heavily polluted soil produced a large effect on the biomass. Additionally, contrary results have been reported in the literature about the auxin effect on heavy metal phytoextraction, depending on the plant species. For example, in one study, *Brassica juncea*, a widely used species for phyotextraction of Pb, did not reveal any increase in its efficiency in the presence of IAA [20]. Considering this finding and our results, the data may be indicating that use of auxin to improve Pb phytoextraction is more viable in slightly polluted soils, but only when certain species are employed.

The auxin and soil Pb concentration effects for *T. minuta* and *B. pilosa* on Pb total extraction and exudate extraction power are shown in Table 3. As mentioned above for Pb concentration in plant organs, the Pb total extraction was not in general affected by auxin treatment. Instead, this was principally related to the Pb concentration in the soil. However, there was one case in which auxin produced an enhancement in Pb total extraction, which was for *B. pilosa* total extraction by the root, with this enhancement being due to a higher root biomass and not to a higher Pb concentration (Table 2). *B. pilosa* also presented a greater total extraction from the highly polluted soil, by the whole plant, compared to *T. minuta*, when plants grew under auxin effects.

Plants grown under a high Pb concentration in soil produced exudates with a higher Pb extraction power than those grown in medium or low treatments. Although auxin did not reveal statistically significant differences, there was a trend of increased exudate extraction power when this growth promoter was used. These results indicate that plants respond to the Pb concentration in soil by modifying the root exudate production, and also probably their composition. The organic compound quantity in exudates should now be examined, as the bibliography indicates a strong relationship between exudate composition with heavy metal solubilizing in soil and extraction by plants [24,25].

3.2. Relationship between exudate extraction power and phytoextraction parameters

Table 4 shows the regression results for the Pb concentration in plant organs and Pb total extraction, with exudate extraction power being expressed as the concentration of Pb in the extraction solution (1 g of soil in 15 mL of extraction solution). The regression parameter R^2 was statistically significant: for Pb concentration in stem and root, and for Pb total extraction by stem, root and whole plant in *T. minuta*; for Pb concentration in root, and for Pb total

Table 3
Mean values ± SD and results of the analysis of variance (ANOVA) comparing Pb total extraction per plant in leaves, stem roots and the whole plant, and the root exudate extraction power for *Tagetes minuta* L. and *Bidens pilosa* L. grown in soils with different Pb concentrations and auxin treatment.

			Pb total extraction (μg per plant)				Exudate extraction power	
Species	Treatment ANOVA	Soil	Leaves ns	Stem ***	Root ***	Plant ***	Concentration (μg mL^{-1}) *	Extraction (μg) *
Tagetes minuta L.	Auxin	Low	2.89 ± 1.58	9.1 ± 3.1 b	1.8 ± 1.4 c	14 ± 5 c	0.05 ± 0.05 b	0.8 ± 0.7 b
		Medium	1.11 ± 1.07	1.21 ± 0.95 b	4.9 ± 1.5 d	7 ± 5 c	0.18 ± 0.09 b	3.2 ± 1.4 b
		High	1.32 ± 1.12	31.8 ± 5.6 a	54 ± 6 b	87 ± 14 b	0.91 ± 0.33 a	16 ± 6 a
	Control	Low	5.87 ± 3.01	7.1 ± 2.9 b	0.8 ± 0.7 e	14 ± 5 c	0.14 ± 0.08 b	2.4 ± 1.3 b
		Medium	3.36 ± 1.75	15.3 ± 10.2 b	7.3 ± 1.6 d	26 ± 6 c	0.09 ± 0.07 b	1.6 ± 1.2 b
		High	3.62 ± 1.85	31.3 ± 5.5 a	53 ± 6 b	88 ± 14 b	0.66 ± 0.21 a	12 ± 4 a
Bidens pilosa L.	Auxin	Low	2.72 ± 1.52	nd	1.2 ± 1.1 e	4 ± 4 c	0.16 ± 0.07 b	2.8 ± 1.3 b
		Medium	0.95 ± 0.81	6.6 ± 2.9 b	13 ± 2 c	21 ± 6 c	0.14 ± 0.08 b	2.5 ± 1.3 b
		High	4.54 ± 2.26	35.3 ± 8.3 a	167 ± 57 a	207 ± 71 a	1.35 ± 0.79 a	24 ± 14 a
	Control	Low	1.02 ± 0.85	8.9 ± 3.1 b	0.7 ± 0.7 e	11 ± 5 c	0.19 ± 0.08 b	3.4 ± 1.4 b
		Medium	4.26 ± 2.13	3.4 ± 2.6 b	14± 2 c	22± 6 c	0.03 ± 0.03 b	0.65 ± 0.51 b
		High	4.33 ± 2.16	28.4 ± 5.1 a	90 ± 17 b	122 ± 22 b	1.04 ± 0.43 a	28.4 ± 5.1 a
Exudate control-blank			–	–	–	–	0.23 ± 0.09 b	4.1 ± 1.5 b

Values in each column followed by the same letter do not differ significantly at $p < 0.05$. (ns, not significant. nd: not detectable. *$p < 0.05$**; $p < 0.01$; ***$p < 0.001$).

Table 4
Simple linear regression results for Pb concentration and total extraction in leaves, stem, root and the complete plant, with the Pb extraction power of root exudates as the regressor variable (X).

Species	Dependent Variable (Y)	Model	R^2	p value
Tagetes minuta	Pb Concentration in stem	Y = 43 X + 9	0.48	<0.01
	Pb Concentration in leaves	–	–	ns
	Pb Concentration in roots	Y = 434 X + 102	0.63	<0.001
	Pb extraction by stem	Y = 22 X + 8	0.37	<0.001
	Pb extraction by leaves	–	–	ns
	Pb extraction by roots	Y = 34 X + 9	0.34	<0.05
	Total Pb extraction per plant	Y = 55 X + 21	0.42	<0.05
Bidens pilosa	Pb Concentration in stem	–	–	ns
	Pb Concentration in leaves	–	–	ns
	Pb Concentration in roots	Y = 487 X + 142	0.5	<0.001
	Pb extraction by stem	Y = 17 X + 5	0.75	<0.001
	Pb extraction by leaves	–	–	ns
	Pb extraction by roots	Y = 95 X + 1.4	0.85	<0.001
	Total Pb extraction per plant	Y = 114 X + 9	0.85	<0.001

extraction by stem, root and whole plant in *B. pilosa*. All these cases presented positive slopes, indicating that root exudates are important for Pb root extraction by the studied species and its translocation to the stem. Nevertheless, the Pb concentration and total extraction in leaves were not related with root exudates, indicating they did not participate in translocation from the stem to leaves.

Related to these results, Shahid et al. [26] reviewed the latest findings concerning this topic, and were able to conclude that the root exudate composition influence on Pb solubilization depends on soil physico-chemical conditions and microbial activity, with this being a function of plant species. With respect to exudate composition influence on Pb uptake by plants and translocation to aerial parts, these authors highlighted a significant increase in Pb root uptake by accumulating and non-accumulating *Sedum alfredii* plants, but with an increased Pb translocation to the shoot being observed only for accumulating *S. alfredii*. However, although the mechanism behind this rise in Pb translocation to shoot tissues by organic compounds present in root exudates is still unknown according to the review, it is proposed that this might be due to the presence of carrier proteins, which bind and translocate Pb to the shoot tissues. Then, once inside the plant roots, carrier proteins can bind this Pb and translocate it to shoot tissue.

From the above considerations, our results suggest that *B. pilosa* and *T. minuta* respond to Pb pollution in soil by modifying their root exudate composition, with this strategy resulting in a higher Pb uptake by roots and translocation to the stem. As the bibliography indicates that root exudate organic compounds only enhance shoot heavy metal concentrations in accumulating plants, then our studied species presented an accumulating behavior.

3.3. Lead extraction efficiency

T. minuta and *B. pilosa* Pb extraction efficiency was calculated by considering Pb TE_{plant} and the mass of soil containing that amount of Pb. In this way, efficiency (Table 5) was expressed as the mass of soil completely remediated (Pb concentration of 25 μg g^{-1}) by one plant. Auxin had no effect on the Pb extraction efficiency of either species, while greater Pb concentration in soil increased *B. pilosa* efficiency. One plant of this species was able to clean up 0.08 g of soil when it grew in medium Pb polluted soil (with or without IAA), and to clean up 0.10 g without auxin and 0.17 g with auxin in high polluted soils. *T. minuta* was able to remediate between 0.03 g and 0.10 g of soil, with no significant differences found among treatments. Other authors performed experiments using widely studied Pb hyperaccumulator plants such as *Sedum alfredii* [27] and *Brassica juncea* [28], and they found values of soil remediation efficiency from 0.3 g in soil under control conditions (no assisted phytoextraction) to 2 g, using several organic compounds that enhanced the uptake rate for *S. alfredii*. For *B. juncea,* an efficiency of 0.015 g of soil was found in both control conditions and using nitrilotriacetate or citric acid. Therefore, we consider that *B. pilosa* and *T. minuta,* despite being species rarely studied for Pb extraction purposes (and only recently found to be Pb accumulators) [13,29], demonstrated an efficiency in control conditions that positioned

Table 5
Lead extraction efficiency of *Tagetes minuta* and *Bidens pilosa* expressed as the mass of soil completely remediated (attaining a Pb concentration of 25 $\mu g\,g^{-1}$) by one plant.

Species	Treatment ANOVA	Soil	Soil Pb_{II} ($\mu g\,g^{-1}$)	TE_{plant} (μg)	Efficiency (g of soil remediated per plant) ***
Tagetes minuta L.	Auxin	Low	25 ± 4	14 ± 5	–
		Medium	296 ± 36	7 ± 5	0.03 ± 0.02 b
		High	1223 ± 256	87 ± 14	0.07 ± 0.03 b
	Control	Low	25 ± 4	14 ± 5	–
		Medium	296 ± 36	26 ± 6	0.10 ± 0.04 a
		High	1223 ± 256	88 ± 14	0.07 ± 0.03 b
Bidens pilosa L.	Auxin	Low	25 ± 4	4 ± 4	–
		Medium	296 ± 36	21 ± 6	0.08 ± 0.03 b
		High	1223 ± 256	207 ± 71	0.17 ± 0.05 a
	Control	Low	25 ± 4	11 ± 5	–
		Medium	296 ± 36	2 ± 6	0.08 ± 0.03 b
		High	1223 ± 256	122 ± 22	0.10 ± 0.05 a

Values in each column followed by the same letter do not differ significantly at $p < 0.05$.

them within the range defined by two important species widely used in Pb phytoremediation.

4. Conclusions

The species studied were sensitive to the application of auxin, but hormones only promoted plant growth when they were not exposed to Pb polluted soils. In addition, the toxic effect of Pb in medium and high polluted soils could not be offset by auxin application. Although total Pb extraction was not generally modified by the use of auxin, *B. pilosa* archieved a higher extraction when grown in highly contaminated soil with this phytohormone applied.

T. minuta and *B. pilosa* produced root exudates capable of extracting Pb when grown in highly polluted soils, indicating a behavioral response to Pb compatible with phytoremediation aims, with the Pb extraction power of root exudates being positively related to Pb uptake and its translocation to the stem. Future work should determine their organic composition to reveal which specific compounds favor uptake and translocation, thereby improving phytoremediation success.

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