



ORIGINAL ARTICLE

Márcio Paulo Pereira¹
Fabricio José Pereira^{1*}
Felipe Fogaroli Corrêa¹
Cynthia de Oliveira¹
Evaristo Mauro de Castro¹
Sandro Barbosa²

¹Universidade Federal de Lavras – UFLA,
Departamento de Biologia, Programa de Pós-
graduação em Botânica Aplicada, Campus
Universitário, 37200-000, Lavras, MG, Brasil
²Universidade Federal de Alfenas – UNIFAL-MG,
Instituto de Ciências da Natureza, Rua Gabriel
Monteiro da Silva, 700, 37130-000, Alfenas, MG,
Brasil

Corresponding Author:
*E-mail: fabriciopereira@dbi.ufla.br

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Lead tolerance during germination and early growth of the Brazilian peppertree and the morpho-physiological modifications

Tolerância ao chumbo em aroeira-salsa na germinação e no crescimento inicial, e modificações morfofisiológicas

ABSTRACT: Woody plants are important for phytoremediation systems, and few species of lead hyperaccumulators are known. We evaluated the lead (Pb) tolerance of *Schinus molle* L. plants and the resulting anatomical and physiological modifications during germination and early growth. Two experiments were conducted to evaluate the response of *S. molle* seeds and plants to exposure to five concentrations of $Pb(NO_3)_2$ (0.0, 0.5, 1.0, 2.0, and 5.0 mM). The seed germination and seedling growth were unaffected, and the accumulation of Pb was higher in the roots than in the shoots. The root and leaf anatomy was significantly modified, but the internal structure exhibited no evidence of toxicity. *S. molle* accumulated Pb to levels close to those of metal-tolerant plants, although the Pb translocation decreased proportionately as the Pb concentration of the solution increased. Thus, *S. molle* accumulated Pb to concentrations above toxic levels and showed only favorable modifications to the anatomical structure and growth, suggesting this species is Pb tolerant and has the potential for use in phytoremediation systems.

RESUMO: Plantas lenhosas são muito importantes para sistemas de fitorremediação e poucas espécies hiperacumuladoras de chumbo são conhecidas. O objetivo deste trabalho foi avaliar a tolerância de *Schinus molle* L. ao chumbo (Pb), em função de modificações anatômicas e fisiológicas durante a germinação e o crescimento inicial. Dois experimentos foram montados para avaliar a exposição de sementes e plantas de *S. molle* a cinco concentrações de $Pb(NO_3)_2$ – 0,0; 0,5; 1,0; 2,0 e 5,0 mM. Ocorreram modificações significativas na anatomia das raízes e folhas sob exposição ao Pb; contudo, a estrutura interna de *S. molle* não apresentou nenhuma evidência de toxicidade. A germinação das sementes e o crescimento das plantas não foram afetados pela exposição ao Pb. O acúmulo de Pb foi maior nas raízes, em comparação com a parte aérea. *S. molle* acumula teores de Pb próximos aos observados em plantas tolerantes a metais, sendo que a translocação de Pb foi reduzida com o aumento da concentração desse elemento na solução. Portanto, *S. molle* acumula Pb em concentrações acima das consideradas tóxicas e demonstra apenas modificações favoráveis na estrutura anatômica e no crescimento. Dessa forma, esta espécie apresenta tolerância ao Pb e pode ter potencial para ser utilizada em sistemas de fitorremediação.

1 Introduction

Environmental pollution by heavy metals is a major problem worldwide (MCINTYRE, 2003). Lead (Pb) is one of the most important of these environmental pollutants because of its frequent utilization in industry (PRASAD, 2004). In the past 20 years, phytoremediation technology has been attracting scientific and economic interest due to its capacity for recovering polluted environments (MCINTYRE, 2003). Phytoremediation is defined as the utilization of plants able to concentrate environmental pollutants, thus converting them to inoffensive molecules (MCINTYRE, 2003).

Trees are often considered important in phytoremediation systems because of their higher biomass production, larger root system, and higher capacity to store heavy metals in comparison to herbaceous plants (CAPUANA, 2011). These characteristics are important for the utilization of plant biomass as an energy source and for recovering the heavy metals absorbed (ZACCHINI et al., 2009).

The tolerance mechanisms of the metal-hyperaccumulator plants to high heavy-metal concentrations have been intensely investigated in recent years. One of the most common assumptions is that the tolerance mechanisms of the plants are related to anatomical and physiological modifications (LUX et al., 2004; PEREIRA et al., 2011; VACULÍK et al., 2012), which are, in turn, related to modifications for the absorption, translocation, chelation, compartmentalization, and biotransformation of these pollutants (RASCIO; NAVARI-IZZO, 2011). Therefore, focusing on the anatomical and physiological modifications produced by the heavy metals is essential for discovering new hyperaccumulator plants. There are very few heavy-metal hyperaccumulator plants, and only approximately 0.2% of angiosperm species are recognized as hyperaccumulators. However, there should be many undiscovered plant species with the capacity to tolerate metals (RASCIO; NAVARI-IZZO, 2011). In particular, few Pb hyperaccumulator species are described (KRÄMER, 2010). Therefore, investigating new species for this capacity is critically important for phytoremediation efforts.

Schinus molle L. (Anacardiaceae), known as the “pepper tree” or “aroeira salsa”, is a tree that produces a large biomass and has a tolerance to drought and heat (IPONGA; MILTON; RICHARDSON, 2008). This species may be useful for phytoremediation systems as it grows quickly and has non-deciduous leaves with a tolerance to different environmental conditions. Although this species is cultivated worldwide for reforestation, the Pb tolerance of *S. molle* has yet to be investigated.

The objective of this study was to evaluate the capacity of this species to tolerate Pb and the possible resulting modifications to germination, early growth, and anatomical structure.

2 Materials and Methods

Seeds from *S. molle* were collected from plants utilized in urban arborization in the southern part of Minas Gerais State in Brazil. The seeds were surface sterilized with a 1.0% sodium hypochlorite solution for 10 min, rinsed in tap water, dried at 35 °C, and stored in paper bags at 4 °C until the experiments

were initiated. Two experiments were conducted: one to evaluate the effects of Pb on *S. molle* germination, and a second to evaluate the effects of Pb on early growth.

The seeds were placed in gerbox-type pots containing vermiculite that had been treated with 90 mL of Pb(NO₃)₂ solutions (0.0, 0.5, 1.0, 2.0, and 5.0 mM) prepared based on the Brazilian Conselho Nacional do Meio Ambiente Law, which determines the different levels for Pb disposal in soils. The substrate was watered daily with distilled water to replace the same volume lost by evapotranspiration. All experiments were conducted in a growth chamber with a photosynthetically active radiation flux of 43 mmol m⁻² s⁻¹ at 25 ± 2 °C.

The experimental design was randomized with five treatments and six replicates, with an experimental unit composed of one gerbox containing 30 seeds. The biometric measurements of the seedlings were performed five weeks after initiating the experiment by measuring the fresh mass of the roots and shoots for three seedlings from each replicate. The root and stem diameters were also measured by digitally photographing the seedlings and analyzing the images with UTHSCSA-ImageTool software from the University of Texas Health Science Center.

For the anatomical analysis, seedling roots were collected and fixed with FAA_{70%} ([70% ethanol:formalin: acetic acid (90:5:5)]) for 72 h and stored in 70% ethanol. Transverse sections were prepared from the maturation zone of the roots, 0.5 cm from the root tip, with a bench-top microtome (LPC type). The sections were stained with an aqueous safrablau solution (0.1% safranin:1% astra-blau, 7:3) and mounted on semi-permanent slides (JOHANSEN, 1940). The slides were examined and photographed on an Axio Scope A1 light microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) equipped with a digital camera. The digital images were evaluated with the UTHSCSA-ImageTool image analysis software. Three histological sections and a total of 45 fields were evaluated for each anatomical characteristic for each treatment. The following anatomical characteristics were evaluated: thicknesses of the root epidermis, endodermis, and exodermis; number of tracheary elements of xylem per unit area; and thickness of the phloem. The vulnerability index was calculated as described by Carlquist (1975).

The second experiment was conducted with young plants produced from the stored seeds described in the plant materials section. The seeds were placed in gerbox-type pots containing vermiculite and 90 mL of distilled water and were watered daily with the same volume lost by evapotranspiration. The seeds were maintained in the growth chamber as described previously until the plants developed a root system and shoots. When the seedlings were 2.0 cm in height, they were transferred to plastic trays containing 3.0 L of vermiculite and 1.8 L of the nutritive solution described by Hoagland and Arnon (1950) at 20% of its ionic force. After acclimatization, the plants were maintained in the greenhouse for 45 days and exposed to the same concentration of Pb(NO₃)₂ solutions used for the germination experiment. The nutritive solution was replaced at five days intervals, and the system was watered daily with the same volume lost by evapotranspiration.

The plants were harvested 15, 30, and 45 days after initiating the experiment. For each harvest, the leaf area was determined

by image analysis with the UTHSCSA-ImageTool software. The plants were segmented into roots, stems, and leaves and dried at 45 °C for 48 h, after which the dry mass was determined for each plant part. The data from the dry mass and leaf area measurements were used to calculate the relative growth rate, leaf area ratio, net assimilation rate, and specific leaf area as described by Hunt et al. (2002). The experimental design was randomized in a 3 × 5 factorial scheme with 10 replicates.

Forty five days after initiating the experiment, the anatomy of the first completely expanded leaf of each plant was analyzed. The leaves were fixed in FAA_{70%} for 72 h and stored in 70% ethanol (JOHANSEN, 1940). Transverse sections were cut from the median region of the leaves with the bench-top microtome (LPC type), clarified with 50% sodium hypochlorite, stained with the aqueous safrablau solution, and mounted on semi-permanent microscopy slides (JOHANSEN, 1940). The slides were examined and photographed on the light microscope, and the digital images were evaluated with the UTHSCSA-ImageTool software. The following anatomical traits were evaluated on the transverse sections: thickness of the leaf epidermis on the adaxial and abaxial surfaces, thickness of the mesophyll, and the vulnerability index as described by Carlquist (1975). Paradermic sections were obtained from the adaxial and abaxial leaf surfaces, clarified with 50% sodium hypochlorite, stained with a 1% aqueous safranin solution, and mounted on microscopy slides (JOHANSEN, 1940). The paradermic sections were evaluated for the stomatal density (number of stomata per mm²) and the stomatal index [number of stomata/(number of stomata + number of common epidermal cells) × 100] was calculated. The experimental design was randomized with five treatments and five replicates. Three sections and 15 fields were evaluated for each replicate.

Sixty days after initiating the experiment, the Pb in the plant tissues was quantified separately for the roots and shoots. Each plant part was dried at 45 °C for 48 h, and 500 mg of dry mass was ground in liquid nitrogen with a mortar and pestle before digesting in 10 mL of HNO₃ for 30 min at 150 °C in a block digestion system. Subsequently, 1.0 mL of HClO₄ was added, and the temperature was elevated to 210 °C for 20 min. The digested material was diluted to a volume 25 mL with distilled water, and the Pb concentration was determined with an atomic absorption spectrometer. The experimental design was randomized with five treatments and three replicates.

The data were subjected to one-way analyses of variance and the means were compared by the Scott-Knott test or a regression analysis, depending on the data adjustment required for the models. All statistical analyses were conducted with Sisvar 5.0 statistical software (FERREIRA, 2011).

3 Results and Discussion

None of the Pb concentrations affected the germination of *S. molle* (Table 1). In some plant species, the effects of heavy metal toxicity on germination may partially be determined by selective ion uptake through the seed coat and external tissues surrounding the embryo (LI et al., 2005). As such, the absence of Pb effects on the germination of *S. molle* may be due to a barrier effect of the seed coat blocking the Pb penetration and protecting the embryo.

No change was seen in the seedling root and colletum diameters with any Pb concentration, nor were the root and shoot length or fresh mass affected (Table 1). Ozdener and Kutbay (2009) reported reductions to the root length of *Eruca sativa* at Pb concentrations of 0.23 mM or higher, and reductions of the shoot length at Pb concentrations greater than 0.30 mM, describing the behavior of the species as sensitive. In contrast, Shaukat, Mushtaq and Siddiqui (1999) showed that the Pb tolerant species *Pennisetum americanum* exhibited no seed germination or seedling growth effects, even when exposed to 0.30 mM Pb(NO₃)₂. Therefore, plant species respond to Pb based on both its concentration and the endogenous tolerance mechanisms of the species. *S. molle* showed a capacity to cope with Pb stress at an even higher concentration of 5.0 mM, and thus, it may be a potential species for phytoremediation.

The exposure of *S. molle* to different Pb concentrations did alter the anatomical structure of the leaves and roots, although no toxicity effects were observed in these organs (Figure 1).

The thickness of the root epidermis increased as the Pb concentration increased (Figure 2a). Compared to the control, the higher Pb concentrations increased the root apoplastic barriers, as can be observed by the thickening of the endodermis and exodermis (Figure 2b). The root vascular tissues of *S. molle* were also modified by the Pb as the concentration increased, with the thickness of the phloem increasing (Figure 2c) and the vulnerability index decreasing (Figure 2d).

One mechanism that reduces the toxicity of Pb for plant hyperaccumulator species is to reduce the heavy metal translocation from the roots to the shoots (KRÄMER, 2010).

Table 1. Germination and biometric characteristics of *Schinus molle* seedlings exposed to different Pb(NO₃)₂ concentrations.

	Pb (mM)					*P-value
	0.00	0.50	1.00	2.00	5.00	
Germination (%)	34.00	27.33	28.00	36.00	31.33	0.34 ^{ns}
Root diameter (mm)	0.73	0.76	0.83	0.91	0.78	0.47 ^{ns}
Colletum diameter (mm)	0.88	0.82	0.87	1.03	0.80	0.14 ^{ns}
Root length (mm)	18.09	15.61	12.72	13.44	14.37	0.22 ^{ns}
Shoot length (mm)	22.65	19.12	20.58	20.84	20.94	0.76 ^{ns}
Fresh mass (mg)	23.78	29.78	31.97	27.38	27.06	0.21 ^{ns}

*5% significance for the F-test. ^{ns}not significant. Pb, lead.

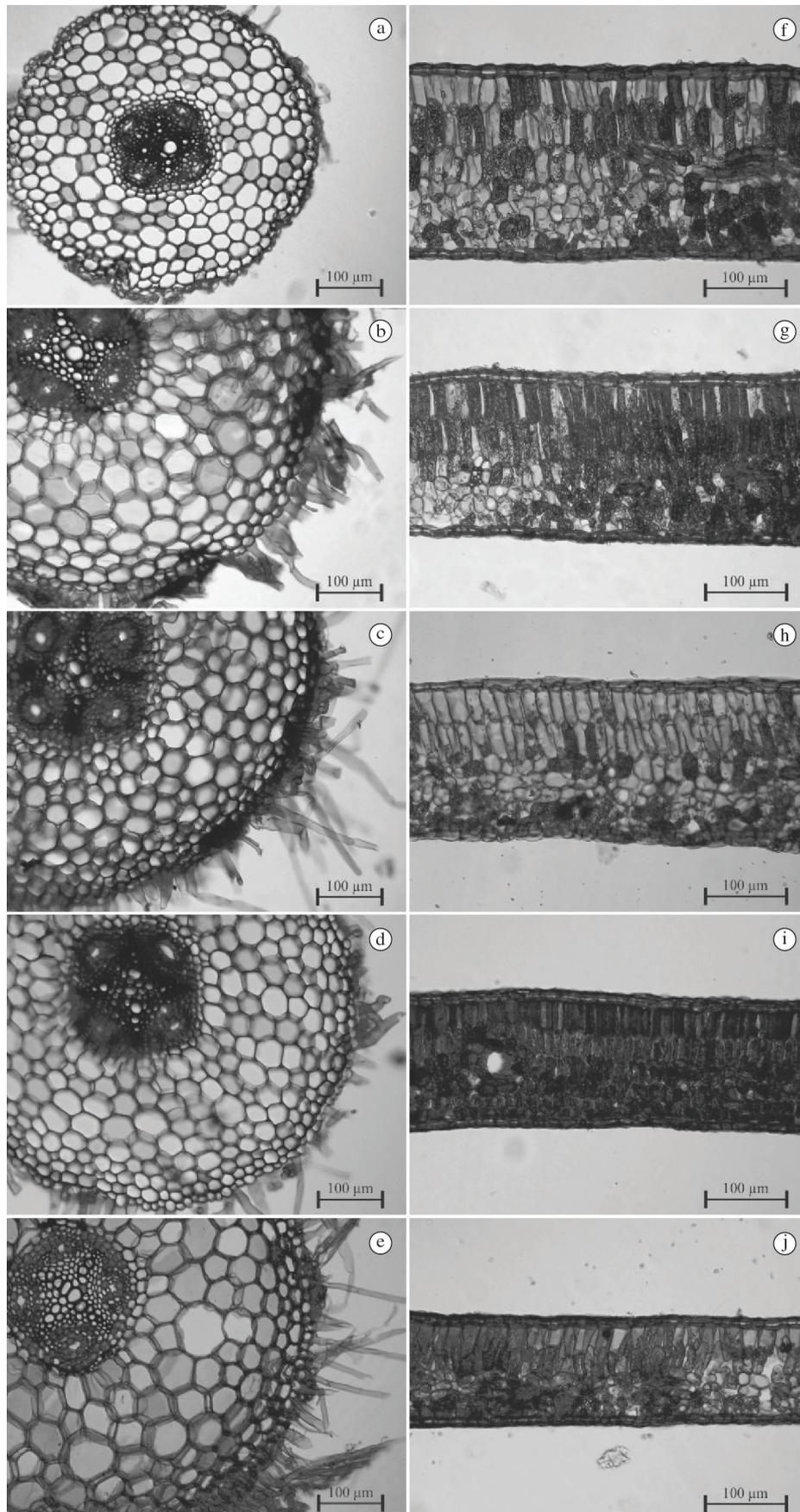


Figure 1. Root anatomical features of seedlings and leaves from young *Schinus molle* plants exposed to increasing concentrations of $Pb(NO_3)_2$. Images: (a, f) control (0.0 mM); (b, g) 0.5 mM; (c, h) 1.0 mM; (d, i) 2.0 mM; (e, j) 5.0 mM.

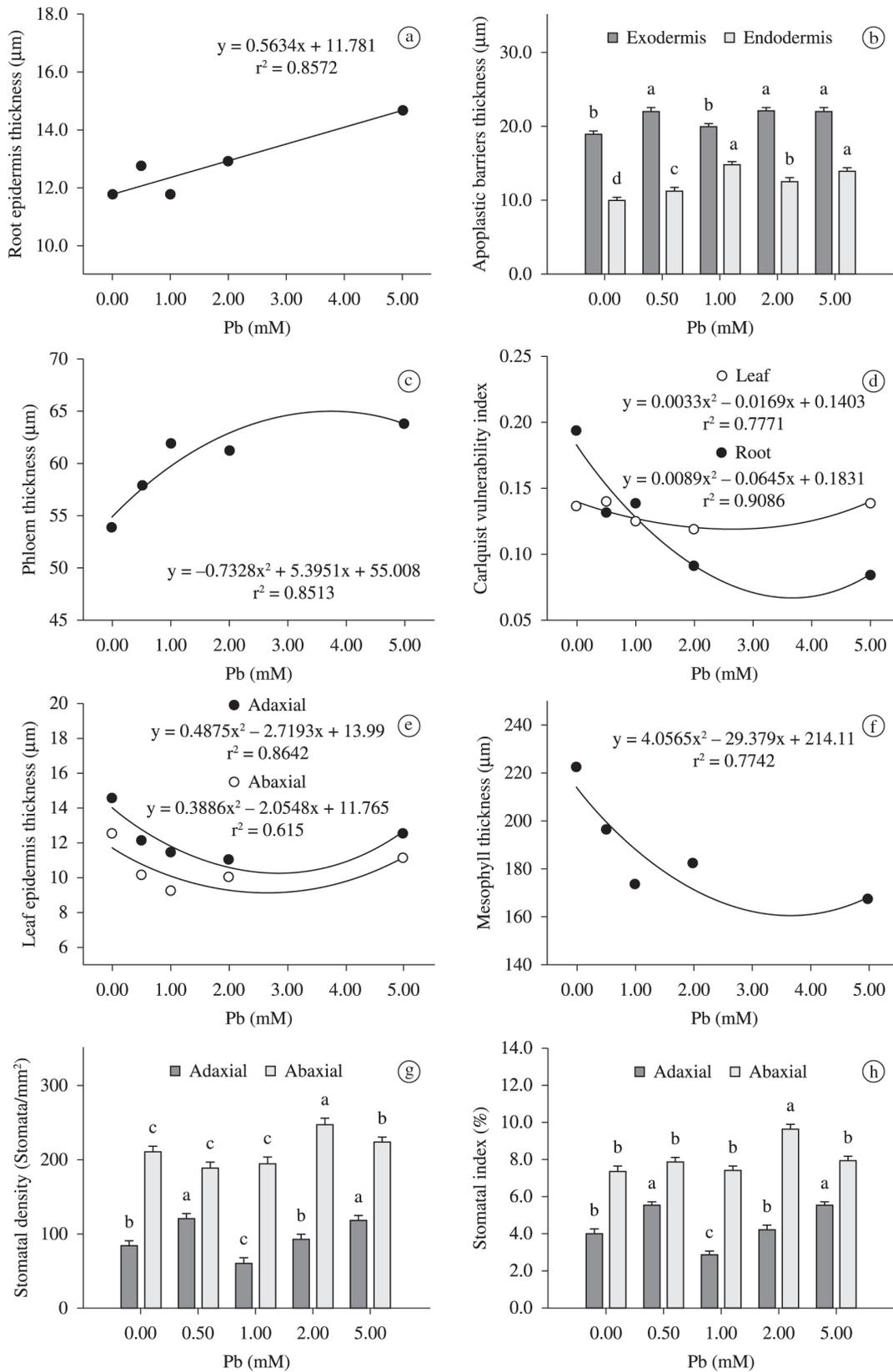


Figure 2. Anatomical characteristics of the seedling roots and young plant leaves from *Schinus molle* plants grown under different $\text{Pb}(\text{NO}_3)_2$ concentration regimes. (a) Mean root epidermal thickness; (b) Mean apoplastic barrier thickness for the root exodermis and endodermis; (c) Mean thickness of the phloem in the roots; (d) Mean vulnerability index as defined by Carlquist (1975) for the leaves and roots; (e) Mean thickness of the adaxial and abaxial leaf epidermis; (f) Mean thickness of the mesophyll; (g) Mean stomatal density of the adaxial and abaxial leaf surfaces; (h) Mean stomatal index for the adaxial and abaxial leaf surfaces. The means designated with the same letters in b, g, and h did not significantly differ ($p < 0.05$) with the Scott-Knott test. Bars in b, g, and h are the standard error of the mean. Pb, lead.

Salix clones with a recognized tolerance to Pb showed a higher proportion of epidermis, exodermis, and endodermis in the roots (LUX et al., 2004), which may have resulted from the reduction in heavy-metal translocation. Likewise, lignin deposition in the apoplastic barriers (exodermis and endodermis) may be associated with the allocation of Pb to the roots (RASCIO; NAVARRI-IZZO, 2011; VACULÍK et al., 2012). Therefore, the increased apoplastic barriers seen in the *S. molle* roots may be an indication of an important toleration mechanism.

The reduction in the vulnerability index could relate to a decreased diameter of the tracheary elements and an increased number of these cells (CARLQUIST, 1975). *Salix caprea* plants exposed to zinc and cadmium contamination exhibited a larger proportion of vascular tissues (VACULÍK et al., 2012), which might increase their capacity for water and nutrient translocation. Heavy metal tolerant species such as *Eichhornia crassipes* develop an increased capacity for conducting water and nutrients due to modifications of the vascular tissues (PEREIRA et al., 2011). Similarly, the anatomical modifications to the vascular tissues of *S. molle* may provide an important Pb toleration mechanism, increasing the capacity for water and nutrient conduction for this species.

Anatomical modifications were also observed in *S. molle* leaves. The epidermal thickness decreased as the Pb levels increased on both the adaxial and abaxial surfaces (Figure 2e), and the thickness of the mesophyll decreased (Figure 2f). Despite this, the stomatal densities and indices (Figure 2g, h) increased with higher Pb concentrations. Reductions in the thickness of the epidermis and mesophyll are common responses by plants growing under conditions of heavy metal contamination. Marques et al. (2011) reported similar results in *Eucalyptus* plants growing in soil contaminated with cadmium. The anatomical modifications of the leaves under conditions of heavy metal contamination could result from physiological modifications (SHI; CAI, 2009; SOUZA et al., 2009). Plant structure and function are closely related, and an increased stomatal density and index could result in a higher capacity for CO₂ uptake (PEREIRA et al., 2011). This higher proportion of CO₂ in the leaves could be fixed by photosynthesis, which might promote plant growth and development. These results could also relate to the lack of modifications to the physiological growth index observed for the *S. molle* plants. The increases in the stomatal index and density are important

anatomical traits for plants growing in Pb-contaminated soils, and *S. molle* had these favorable leaf anatomical modifications.

The total amount of Pb accumulated in the plant biomass increased proportionally with the increasing Pb concentrations (Figure 3a). At the higher concentrations, the maximum values for the Pb in the plants were approximately 1000 mg kg⁻¹, and the accumulation in the plant roots was three times higher than that in the shoots. Although these plant parts showed an increased accumulation of Pb under the conditions of maximum Pb concentration (Figure 3b), the proportion of the Pb translocated was reduced as the concentration of the metal increased (Figure 3c).

Lead has been shown to have low mobility in the plant body, and most tolerant plant species store this element in the roots (SHARMA; DUBEY, 2005). Most of the Pb being stored in the plant roots may be caused by thickening of the apoplastic barriers reducing the translocation of the metal. Krämer (2010) established the critical level of Pb as 0.6-28.0 mg kg⁻¹, but Pb hyperaccumulator plants contain approximately 1000 mg kg⁻¹ of Pb in the plant tissues. *S. molle* can also cope with the toxicity effects of Pb levels up to the critical concentrations. Herbaceous hyperaccumulator plants may tolerate the high Pb concentrations in their tissues, but the biomass production of these plants is very low in comparison to the woody plants and trees (CAPUANA, 2011). Because of their greater biomass, growth, and ability to permanently retain the Pb, tolerant trees such as *S. molle* are important for phytoremediation systems.

The plant physiological growth indices were not affected by the Pb concentrations (Table 2), but the time of exposure affected the relative growth rate ($F = 2.987$; $p = 0.0554$), leaf area ratio ($F = 7.515$; $p = 0.0010$), net assimilatory rate ($F = 6.665$; $p = 0.0020$), leaf area ($F = 11.811$; $p < 0.01$), and plant dry mass ($F = 37.956$; $p < 0.01$). Although increasing exposure reduced the plant growth characteristics, the leaf specific area was unaffected by the Pb concentrations ($F = 1.852$; $p = 0.1629$) or the length of time of exposure ($F = 1.070$; $p = 0.3759$).

A portion of plant tolerance to heavy metals can be defined by its capacity to maintain its survival rate and growth in environments containing high concentrations of these elements (RASCIO; NAVARRI-IZZO, 2011). The capacity of *S. molle* to maintain its growth characteristics under all of the Pb concentration conditions is an important characteristic for classifying this plant as a tolerant species. A reduction in the plant growth characteristics over time is a natural response

Table 2. Early growth of young *Schinus molle* plants under different Pb(NO₃)₂ concentrations.

	Pb (mM)					*P-value
	0.00	0.50	1.00	2.00	5.00	
PDM (mg)	106.19	109.52	89.52	99.05	84.29	0.15 ^{ns}
LA (cm ²)	7.66	8.11	6.73	7.54	7.18	0.59 ^{ns}
RGR (g g ⁻¹ d ⁻¹)	0.032	0.035	0.029	0.032	0.038	0.53 ^{ns}
NAR (g cm ⁻² d ⁻¹)	3.041	4.178	3.209	3.504	4.019	0.17 ^{ns}
LAR (cm ² g ⁻¹)	098.08	113.24	103.74	109.39	107.24	0.14 ^{ns}
SLA (cm ² g ⁻¹)	175.37	238.68	177.29	184.48	176.37	0.38 ^{ns}

*5% significance level for the F-test. ^{ns}not significant. Pb, lead; PDM, whole plant dry mass; LA, leaf area; RGR, relative growth rate; NAR, net assimilatory rate; LAR, leaf area ratio; SLA, specific leaf area.

for any plant species, and this behavior is promoted by the increased levels of energy utilized for maintaining basic metabolic activity (BENINCASA, 2003).

The leaf specific area for photosynthesis is defined by Benincasa (2003) as the leaf area ratio. This characteristic was

not modified by the Pb exposure, and the photosynthetic area was preserved without toxic effects on the *S. molle* plants. The reduction of the leaf area ratio over time is a natural response and may be related to the higher leaf area ratio during the early plant developmental stages (BARREIRO et al., 2006)

The *S. molle* plants also displayed the capacity to maintain their net assimilatory rate under all of the Pb concentration regimes, which is another important characteristic for a hyperaccumulator species related to the maintenance of net photosynthesis by the plant. During the early plant growth stages, growth rates are increased, and then they are reduced over time, which promotes higher respiratory rates and leaf shadowing (AIRES; SILVA; EICHOLZ, 2011).

The leaf specific area is an index of the plant response to the environmental modifications and increases in value when plants are stressed. The *S. molle* plants did not exhibit any change in their leaf specific areas in the presence of Pb, which may be interpreted as the plants not being stressed and the leaf area and biomass being preserved. Thus, all of the physiological growth indices evaluated indicate *S. molle* plants have the characteristics to tolerate Pb contamination.

4 Conclusions

S. molle tolerates and accumulates high Pb concentrations above the limits of nontolerant species. This species showed only beneficial modifications to its anatomical structure under the conditions of Pb contamination without any evidence of phytotoxic effects on the plant structure, and the germination and early growth of the plants were unaffected. All of the characteristics evaluated in this study indicate that *S. molle* is tolerant to Pb contamination and may have the potential for use in phytoremediation and forest restoration areas.

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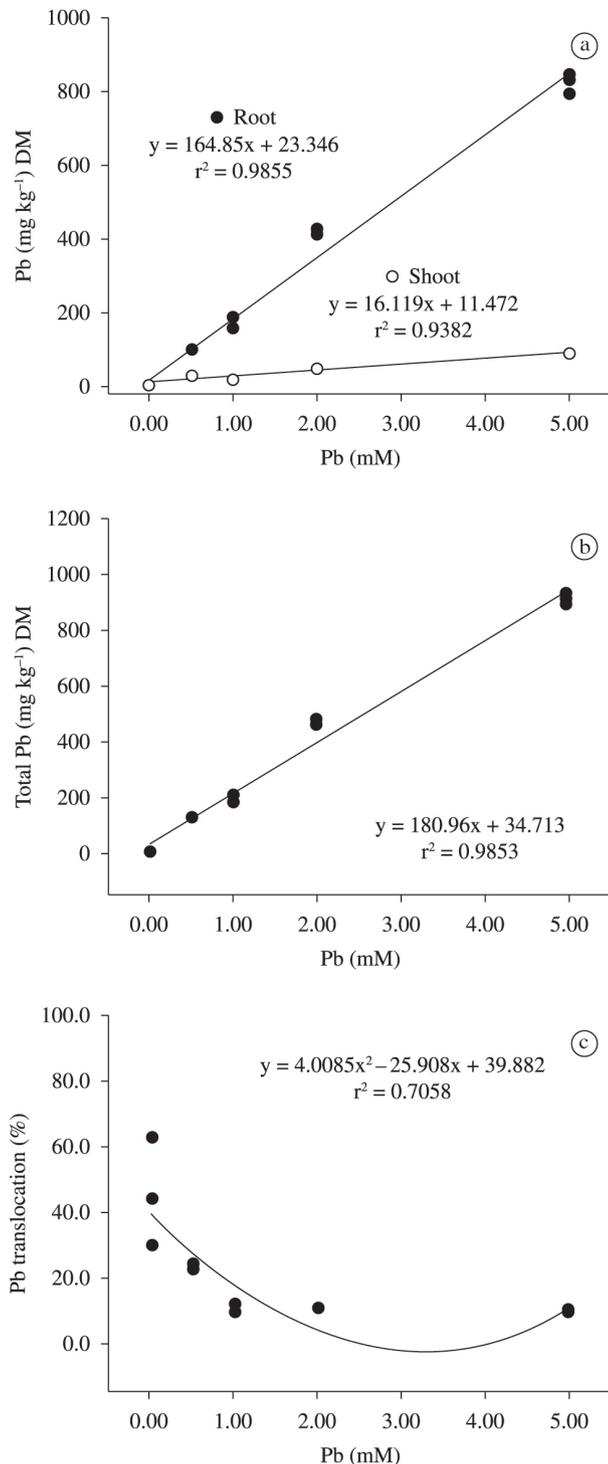


Figure 3. Accumulation and translocation of lead (Pb) in *Schinus molle* plants growing under different Pb(NO₃)₂ concentration regimes. (a) Mean concentration of Pb in roots and shoots; (b) Mean concentration of Pb in the total plant; (c) Mean proportion of the Pb translocated into the plant. DM, dry mass.

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