

Lead tolerance and accumulation in initial sporophytes of *Regnellidium diphyllum* Lindm. (Marsileaceae)

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Abstract

Regnellidium diphyllum Lindm. is a heterosporous fern which grows in shallow waters and wetlands, and water pollution contributes to its vulnerability. Environmental lead contamination is mostly caused by industrial and agricultural residues as well as domestic sewage. Given its persistence in the environment, lead can cause important toxicity in living organisms. Megaspore germination and the initial growth of *R. diphyllum* sporophytes were assessed in Meyer's solution with lead nitrate ($\text{Pb}(\text{NO}_3)_2$) concentrations of 0 (control), 1, 5, 10 and 50 mg L^{-1} . The study was conducted in a growth chamber at $25 \pm 1^\circ\text{C}$ and a 12 hour photoperiod with a nominal irradiance of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, for 28 days. Lead concentration in sporophytes was assessed using atomic absorption spectrometry. In the absence of lead, 74% of spores germinated, while significantly lower germination percentages were observed in $\text{Pb}(\text{NO}_3)_2$ concentrations of 1, 10 and 50 mg L^{-1} . The presence of lead did not significantly influence root growth. At 28 days, primary leaf development was significantly lower in $\text{Pb}(\text{NO}_3)_2$ concentrations of 5 mg L^{-1} and higher in relation to the control. The length of secondary leaves did not significantly differ between sporophytes exposed to different concentrations of lead and those of the control at 28 days. Sporophytes exposed to 10 and 50 mg L^{-1} $\text{Pb}(\text{NO}_3)_2$ accumulated 1129 mg kg^{-1} and 5145 mg kg^{-1} of Pb, respectively. The presence of high levels of lead in *R. diphyllum* sporophytes did not prevent initial development. Future studies should investigate the ability of the species to accumulate and tolerate high levels of lead in advanced stages of its development and in environmental conditions.

Keywords: wetlands, species conservation, fern, heavy metals, pollutant.

Tolerância e acumulação de chumbo em esporófitos jovens de *Regnellidium diphyllum* Lindm. (Marsileaceae)

Resumo

Regnellidium diphyllum Lindm. é uma samambaia heterosporada que se desenvolve em águas rasas ou em solos úmidos, sendo que a poluição da água contribui para sua vulnerabilidade. A contaminação ambiental por chumbo ocorre principalmente por resíduos industriais e agrícolas, bem como por efluentes domésticos. Devido à sua persistência no ambiente, esse metal pode apresentar importante toxicidade aos organismos vivos. A germinação de megásporos e o desenvolvimento inicial de esporófitos de *R. diphyllum* foram avaliados em solução de Meyer com concentrações de 0 (controle), 1, 5, 10 e 50 mg L^{-1} de nitrato de chumbo ($\text{Pb}(\text{NO}_3)_2$). O estudo foi conduzido em câmara de germinação a $25 \pm 1^\circ\text{C}$ e fotoperíodo de 12 horas sob irradiância nominal de $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, por 28 dias. A concentração de chumbo em esporófitos foi analisada por espectrometria de absorção atômica. Na ausência de chumbo, 74% dos esporos germinaram, enquanto que porcentagens de germinação significativamente menores foram observadas nas concentrações de 1, 10 e 50 mg L^{-1} de $\text{Pb}(\text{NO}_3)_2$. A presença de chumbo não influenciou significativamente o crescimento das raízes. O desenvolvimento das folhas primárias foi significativamente menor em relação ao controle a partir de 5 mg L^{-1} de $\text{Pb}(\text{NO}_3)_2$ aos 28 dias. O comprimento das folhas secundárias não diferiu significativamente entre esporófitos expostos às diferentes concentrações de chumbo e aqueles do controle, aos 28 dias. Esporófitos expostos a 10 e 50 mg L^{-1} de $\text{Pb}(\text{NO}_3)_2$ acumularam 1129 mg kg^{-1} e 5145 mg kg^{-1} de Pb, respectivamente. A presença de altas concentrações de chumbo nos esporófitos de *R. diphyllum* não impediu seu desenvolvimento inicial. Estudos futuros deverão investigar a capacidade de a espécie acumular e tolerar altas concentrações de chumbo em estádios avançados de desenvolvimento e também em condições ambientais.

Palavras-chave: áreas úmidas, conservação de espécies, samambaia, metais pesados, poluente.

1. Introduction

Environmental contamination by heavy metals is mostly caused by improper disposal of industrial and agricultural waste, and by domestic sewage. Most metals are persistent and accumulate in aquatic and terrestrial environments, posing a threat to biodiversity and to human health (Sharma and Dubey, 2005; Mishra and Tripathi, 2008; Hu et al., 2010).

Lead (Pb) is a naturally occurring heavy metal in the environment, and is produced as a result of rock fragmentation. Other sources of lead in water and soil are the mining, welding and steel production industries, effluents of domestic sewage (Gonçalves et al., 2009; García-Lestón et al., 2010), incomplete fossil fuel burning (Paoliello and Chasin, 2001), as well as agricultural inputs (Sharma and Dubey, 2005; Hu et al., 2010).

In the 1970s, gasoline combustion was an important source of air pollution by lead, as tetraethyl lead was added to gasoline at concentrations of approximately 0.4 g L⁻¹ (Paoliello and Chasin, 2001; Sharma and Dubey, 2005). In Brazil, although this additive has been replaced with ethanol since 1993, significant levels of lead are still present in ecosystems due to the persistence of this metal in the environment (Paoliello and Chasin, 2001; Gonçalves et al., 2009).

Lead is relatively abundant in the Earth's crust, and its mean concentration in the soil is approximately 20 mg kg⁻¹ (WHO, 1995). In Brazil, reference values of lead in soils with different uses have been established by the Brazilian National Environmental Council (CONAMA), and range from 180 to 900 mg kg⁻¹ (Brasil, 2009). According to this Council, the legal limit for lead in freshwater classes 1 and 2 is 0.01 mg L⁻¹, while the legal limit for lead in class 3 fresh water is 0.033 mg L⁻¹ (Brasil, 2005).

In plants, lead concentration ranges from 1 to 3 mg kg⁻¹ in leaves, although higher levels may be found in roots (Wallace and Wallace, 1994). The excess of lead can cause toxicity symptoms such as growth reduction, chlorosis, inhibition of photosynthesis, and changes in mineral nutrition and in the availability of growth factors. Plants that survive in lead contaminated soil may present morphological, physiological and biochemical alterations, and may develop mechanisms for detoxification and tolerance, such as accumulating lead in the vacuoles and cell walls of roots and aerial parts (Sharma and Dubey, 2005; Mishra and Tripathi, 2008; Gonçalves et al., 2009).

Tolerance and accumulation of lead in plant tissues have been studied in several macrophytes (Guilizzoni, 1991; Mishra and Tripathi, 2008; Hu et al., 2010). Bioaccumulation of lead has been detected in macrophytes such as the aquatic ferns *Marsilea minuta* L. (Marsileaceae) (Kumar et al., 2012) and *Azolla filiculoides* Lam. (Salviniaceae) (Oren Benaroya et al., 2004; Khosravi et al., 2005).

In some regions of the state of Rio Grande do Sul, there is evidence of water and soil pollution due to the frequent use of chemical products in large areas of wetlands used

for irrigated rice cultivation. Approximately 73% of the 1.3 million hectares of irrigated rice lands in Brazil are in Rio Grande do Sul. The state is also responsible for the consumption of approximately 20% of agrochemicals applied on crops in Brazil (Primel et al., 2005; Silva et al., 2011).

Regnellidium diphyllum Lindm. is a heterosporous fern in the Marsileaceae family. Its occurrence is limited, and populations have been reported in Southern Brazil and in some neighboring areas in Uruguay and Argentina (Schultz, 1949; Alonso-Paz and Bassagoda, 2002). The species grows in shallow waters and frequently flooded wetlands. Adult sporophytes of *R. diphyllum* have a rhizomatic stem which is attached to the surface of the soil or to muddy areas, and have long petioles with bilobed leaves. When in an aquatic environment, leaf blades float on the water surface (Schultz, 1949). The reproductive structures consist of microspores and megaspores contained within sporocarps (Mahlberg and Baldwin, 1975). Currently, *R. diphyllum* is considered an endangered species in Rio Grande do Sul, and is classified as vulnerable (Rio Grande do Sul, 2003). The species' sensitivity to pollutants has only recently begun to be investigated (Wunder et al., 2009; Kieling-Rubio et al., 2010, 2012; Droste et al., 2010; Cassanego et al., 2010, 2013). The present study aimed to assess the influence of lead on germination and on the initial development of *R. diphyllum*, so as to contribute to the understanding of the impact of this heavy metal on the establishment of young sporophytes.

2. Material and Methods

Mature sporocarps were collected from plants in a natural population of *Regnellidium diphyllum* in the municipality of Gravataí (29°57'18"S, 51°1'52"W), in the state of Rio Grande do Sul, Brazil. Voucher specimens were deposited at the Herbarium Anchieta (PACA), São Leopoldo, Brazil.

After being rinsed in running water, 35 sporocarps were placed in a laminar flow chamber and sterilized with 70% ethanol for 30 seconds and 7% sodium hypochlorite for 10 minutes. Sporocarps were then rinsed four times in sterile distilled water and dried on sterile filter paper, after which they were mechanically cracked, and underwent manual separation of megaspores from microspores under a stereo microscope. Megaspores from different sporocarps were mixed to obtain a homogeneous sample. As apogamy is common in *R. diphyllum* megagametophytes (Mahlberg and Baldwin, 1975), the exclusive use of megaspores prevented the establishment of cultures containing a mixture of sexual and apogamous sporophytes (Wunder et al., 2009).

2.1. Experiment I

For megaspore germination and sporophyte development Meyer's solution (Meyer et al., 1955) with pH set to 6.0 before sterilization was prepared as culture medium.

Lead nitrate ($\text{Pb}(\text{NO}_3)_2$) was added to sterile culture media at concentrations of 1, 5, 10 and 50 mg L^{-1} . Control cultures were grown in Meyer's medium without $\text{Pb}(\text{NO}_3)_2$. Fifteen megaspores were placed in each glass vial (4.5×10 cm) with 30 mL of Meyer's solution. Six repetitions were performed for each concentration of $\text{Pb}(\text{NO}_3)_2$. The experiment was conducted in a growth chamber at temperature of $25 \pm 1^\circ\text{C}$, with a 12 hour photoperiod under fluorescent light with a nominal irradiance of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, which are appropriate abiotic conditions for *in vitro* culture of *Regnellidium diphyllum* (Wunder et al., 2009; Droste et al., 2010).

Megaspores were considered to have germinated when an apical globular green structure with a crown of rhizoids was observed (Wunder et al., 2009). Sporophyte development was assessed by removing three randomly selected specimens from each vial at 14 and 28 days of culture, for a total of 18 specimens for each concentration of $\text{Pb}(\text{NO}_3)_2$. The lengths of primary and secondary leaves and of primary roots were measured for each specimen. The occurrence of chlorosis and necrosis of sporophytes was observed and recorded throughout the study.

Data were tested for normality using the Shapiro-Wilk test. Germination percentages were compared using the Kruskal-Wallis test followed by the Student-Newman-Keuls test, with a significance level of 5%. Mean lengths of primary and secondary leaves and of roots were compared using ANOVA followed by the Tukey test, with a significance level of 5%. Analyses were conducted using the BioEstat 5.0 and SPSS 20 software packages.

2.2. Experiment II

To analyze the concentration of lead in sporophytes, 50 megaspores were placed in each glass vial (4.5×10 cm) with 30 mL of Meyer's solution, prepared in the same way as in Experiment I. Ten flasks (500 megaspores) were prepared for the control (no lead) condition and for $\text{Pb}(\text{NO}_3)_2$ concentrations of 10 and 50 mg L^{-1} . The experiment was triplicated for each condition (1500 megaspores). Cultures were kept under the same abiotic conditions described for Experiment I. At 28 days, all sporophytes were washed thoroughly in distilled water, dewatered and then oven dried at 50°C until weight was constant.

Due to their small size and weight, the spores from ten flasks were mixed and used as a single sample for each treatment. Samples from each treatment were analyzed in triplicate by an atomic absorption spectrophotometer in a graphite oven (AAS 110, Varian) to assess the concentration of lead accumulated in the sporophytes. The detection limit for lead using atomic absorption spectroscopy (AAS) was $0.0015 \text{ mg kg}^{-1}$ using a standard Titrisol® (Merck) solution.

Data normality was tested using the Shapiro-Wilk test. The mean concentration of lead in sporophytes was compared between treatments by ANOVA followed by the Dunnett test, with a level of significance of 5%. Analyses were conducted using the SPSS 20 software package.

3. Results

3.1. Experiment I

Regnellidium diphyllum megaspore germination occurred under all concentrations of lead nitrate ($\text{Pb}(\text{NO}_3)_2$) and in the control condition. Seventy four percent of spores germinated in the absence of lead, while germination was significantly lower for spores at $\text{Pb}(\text{NO}_3)_2$ concentrations of 1, 10 and 50 mg L^{-1} ($H=15.4589$, $p=0.0038$) (Figure 1).

Most germinated spores developed sporophytes with a primary root, a primary leaf with linear blade and one to three secondary leaves with bilobed blades. Root growth was not significantly affected by the presence of lead at 14 ($F=2.051$, $p=0.094$) and 28 days of exposure ($F=1.223$, $p=0.307$) (Figures 2a-b). However, signs of toxicity such as necrosis were observed in the roots of sporophytes growing in higher concentrations of $\text{Pb}(\text{NO}_3)_2$.

Starting at $\text{Pb}(\text{NO}_3)_2$ concentrations of 1 mg L^{-1} at 14 days of exposure ($F=16.489$, $p<0.001$) and 5 mg L^{-1} at 28 days of exposure ($F=8.813$, $p<0.001$), the development of primary leaves was significantly lower in experimental conditions than in the control condition (Figures 2c-d).

At 14 days, secondary leaves exposed to a $\text{Pb}(\text{NO}_3)_2$ concentration of 50 mg L^{-1} developed significantly less than the leaves of sporophytes grown in the control condition and in media with lower concentrations of lead ($F=8.741$, $p<0.001$) (Figure 2e). At 28 days, the length of secondary leaves of sporophytes grown in media with concentrations of $\text{Pb}(\text{NO}_3)_2$ did not significantly differ from leaf length in the control condition. However, secondary leaves were significantly longer in 1 mg L^{-1} than in 5 mg L^{-1} of $\text{Pb}(\text{NO}_3)_2$ ($F=3.122$, $p=0.019$) (Figure 2f).

3.2. Experiment II

Chemical analysis indicated significant differences in the concentrations of lead in sporophytes exposed and not exposed to $\text{Pb}(\text{NO}_3)_2$ ($F=56.022$, $p<0.001$). Sporophytes in $\text{Pb}(\text{NO}_3)_2$ concentrations of 10 mg L^{-1} accumulated 1129 mg kg^{-1} , while spores exposed to 50 mg L^{-1} accumulated 5145 mg kg^{-1} of lead. A total of 11 mg kg^{-1} of lead was detected in sporophytes in the control condition.

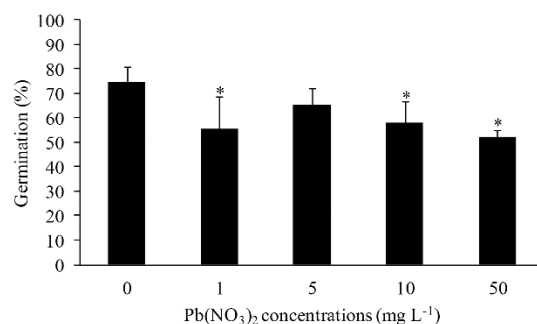


Figure 1. Germination of *Regnellidium diphyllum* megaspores in Meyer's solutions containing a range of lead concentrations at 28 days. Asterisk indicates significant difference between the treatment and the control (0 mg L^{-1}) according to the Student-Newman-Keuls test, $p<0.05$. Error bars indicate standard deviations.

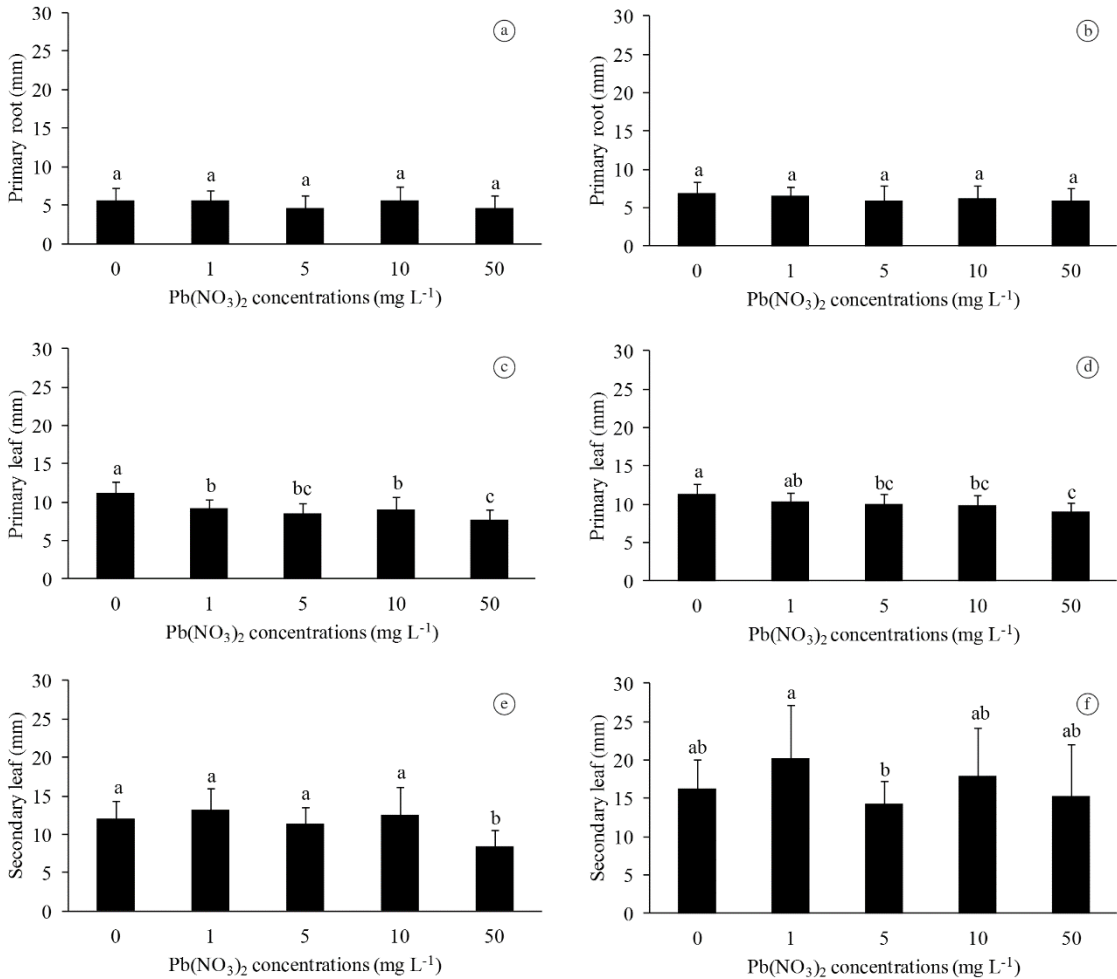


Figure 2. Growth of *Regnellidium diphyllum* in Meyer's solutions containing a range of lead concentrations. a. Primary root length at 14 days. b. Primary root length at 28 days. c. Primary leaf length at 14 days. d. Primary leaf length at 28 days. e. Secondary leaf length at 14 days. f. Secondary leaf length at 28 days. Means with different letters are significantly by the Tukey test, $p < 0.05$. Error bars indicate standard deviations.

4. Discussion

Although *Regnellidium diphyllum* was able to germinate in the presence of 50 mg L⁻¹ Pb(NO₃)₂, there was a 30% reduction in germination percentage compared to the control condition. The terrestrial fern *Athyrium yokoscense* (Franch. & Sav.) Christ presented a comparable reduction in spore germination rates in the presence of 27 mg L⁻¹ of lead (Kamachi et al., 2005). Contrary, the angiosperm *Brassica pekinensis* Rupr. presented a germination percentage of 100% even in a medium containing 125 mg L⁻¹ of lead, and significantly lower percentages were observed only at or above 250 mg L⁻¹ of this metal (Xiong, 1998).

The present results are also similar to findings from studies of *in vitro* exposure of *Regnellidium diphyllum* to other metals. Wunder et al. (2009) found that exposure to 50 mg L⁻¹ cadmium led to a significant reduction in megaspore germination, which was as low as 58%. Exposure to the same concentration of hexavalent chromium also

caused a significant decrease in megaspore germination, which was approximately 25% (Kieling-Rubio et al., 2010). Increasing concentrations of nickel (0.05 to 100 mg L⁻¹) were also associated with a decrease in germination percentage from 75 to 45% (Kieling-Rubio et al., 2012). Lower germination percentages (of approximately 49%) were also observed in megaspores exposed to 50 mg L⁻¹ copper (Cassanego et al., 2013).

In the present study, the development of *Regnellidium diphyllum* sporophytes was not clearly affected by exposure to lead. There was no decrease in root length in any of the lead concentrations tested. Although the primary leaf length was shorter in lead concentrations of 1 (14 days) and 5 mg L⁻¹ (28 days), reduction in the length of the secondary leaf were only observed in the highest concentration tested at 14 days, an observation which was not confirmed at 28 days of exposure. The primary leaf enters into a natural stage of senescence after the first weeks

of the sporophytic phase, and is replaced by secondary bilobed leaves, characteristic of the adult plant. The fact that secondary leaf development was not influenced by the presence of lead suggests that the plants may have developed tolerance to the levels of lead tested, which may not occur in other fern species. On the other hand, Khosravi et al. (2005) observed a 25% growth reduction in adult plants of the aquatic fern *Azolla filiculoides* Lam. exposed for 15 days to an aqueous solution containing 4 mg L⁻¹ Pb(NO₃)₂, suggesting that the species is sensitive to relatively lower concentrations of lead. However, higher concentrations of lead than those tested in the present study, which can be observed in highly polluted soil (Verma and Dubey, 2003), have proved to be toxic to a number of species. The aquatic angiosperm *Myriophyllum spicatum* L. presented a 50% loss in root biomass and a significant reduction in the length of aerial parts when grown in a medium containing 363 mg L⁻¹ lead (Pb²⁺) (Guilizzoni, 1991). A significant gradual reduction in the length of the roots and aerial parts of *Brassica pekinensis* Rupr. seedlings grown in aqueous Pb(NO₃)₂ solutions has also been observed in concentrations at or above 125 mg L⁻¹ of leaf (Xiong, 1998). Lead (Pb²⁺) concentrations of 207.2 mg L⁻¹ also had a negative impact on the growth of *Oryza sativa* L., leading to a 40% reduction in root length and a 31% reduction in the length of the aerial part after 20 days of *in vitro* culture (Verma and Dubey, 2003). The presence of excess lead poses a risk for plants in aquatic and terrestrial environments, as the metal may inhibit the physiological activity of enzymes responsible for plant germination and growth (Sharma and Dubey, 2005).

The negative impact of the exposure of *Regnellidium diphyllum* sporophytes to other metals in nutrient solution was higher when compared to the data obtained in this study. Growth of the root and primary leaf was significantly reduced and there was no development of secondary leaves on sporophytes cultivated in cadmium concentrations of 12.5 mg L⁻¹ and higher (Wunder et al., 2009). Hexavalent chromium was toxic for this species at concentrations at or above 3.2 mg L⁻¹, leading to reduction in root and leaf growth (Kieling-Rubio et al., 2010). The length of roots, primary and secondary leaves was also shorter in sporophytes exposed to nickel concentrations of 3.2 and 4.8 mg L⁻¹ than in sporophytes not exposed to nickel (Kieling-Rubio et al., 2012). Cassanogo et al. (2013) observed significant negative effects on the development of *R. diphyllum* sporophytes grown in media with copper concentrations of 5 mg L⁻¹ and higher.

The visible symptoms of root necrosis in cultures exposed to higher concentrations of lead can be at least partially explained by the excess of lead in the nutrient medium, which can lead to reduction in plant growth, inhibition of photosynthesis, cell injury and tissue necrosis, and might even be lethal (Sharma and Dubey, 2005).

The amount of lead absorbed and accumulated by sporophytes grown for 28 days in a medium containing lead was proportional to the concentrations of lead to which they were exposed. The concentration of lead in

these sporophytes was, respectively, 100 and 500 times the concentration of the metal in the control sporophytes. Similar results have been obtained for the aquatic fern *Azolla filiculoides*, which presented concentrations of lead approximately 620% higher in specimens exposed to solution containing 20 mg L⁻¹ lead for four days (Oren Benaroya et al., 2004) and for the terrestrial fern *Athyrium yokoscense*, which was found to accumulate 23000 mg kg⁻¹ of lead in gametophytes grown for three weeks in solutions containing 2 mg L⁻¹ Pb(CH₃COO)₂ (Kamachi et al., 2005).

The fact that lead was also detected in sporophytes in the control condition may be suggestive of contamination of the plant's natural environment. The area where sporocarps were collected in the Gravataí River Basin is used for agriculture, and particularly irrigated rice cultivation (Rio Grande do Sul, 2013). Therefore, significant amounts of fertilizer, herbicide and fungicide residues may be released in the environment, contributing to lead accumulation in plant reproductive structures (Kamachi et al., 2005; Hu et al., 2010). Furthermore, this area is near a national highway with heavy traffic, which has connected the north coast of the state of Rio Grande do Sul to its capital, Porto Alegre, since it was built in 1973 (CONCEPA, 2013). Traffic on the highway may also have contributed to lead accumulation in the soil, as this metal was added to fuels between the 1970s and the 1990s, and its persistence in the environment is long (Paoliello and Chasin, 2001; Gonçalves et al., 2009).

Lead was also detected in sporophytes of other fern species in natural environments. Kumar et al. (2012) observed 1.02 mg kg⁻¹ of lead accumulated in the dry mass of the aerial parts of *Marsilea minuta* L. sporophytes collected in a humid area near a leather technology park in India. Lead accumulation of 240 mg kg⁻¹ have also been detected in the dry biomass of *Athyrium yokoscense* spores collected from sporophytes grown in polluted soil (Kamachi et al., 2005).

Although the presence of lead negatively influenced megaspore germination, it was not a limiting factor for the initial growth of *Regnellidium diphyllum* sporophytes in the experimental conditions and concentrations tested. The limitation of plant growth observed in studies using polluted media may serve as an indicator of tolerance of each species to different pollutants (Khosravi et al., 2005). Even in *in vitro* studies, in which plants are exposed to predefined concentrations of lead, the accumulation and toxic effects of the metal can vary depending on the species. The tolerance observed in some plants may be related to their physiology, the pH of the medium, tissue permeability and the protection of the reproductive structures (Sharma and Dubey, 2005; Soudek et al., 2010). Some plants develop defense mechanisms, capturing the metal excess in vacuoles or in cell walls (Sharma and Dubey, 2005).

The presence of high concentrations of lead in *Regnellidium diphyllum* sporophytes did not inhibit their initial development, pointing that this species may present tolerance to this metal even in more advanced stages of its life cycle. A species may be considered a lead hyperaccumulator

when it accumulates at least 1000 mg kg⁻¹ of the metal in its dry matter (Baker et al., 1994).

Future studies should investigate the ability of the species to accumulate and tolerate high concentrations of lead in advanced stages of its development and in environmental conditions.

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