Acta Botanica Brasilica 27(1): 26-30. 2013.

Germination and sporophytic development of *Regnellidium diphyllum* Lindm. (Marsileaceae) in the presence of copper

Mara Betânia Brizola Cassanego[1,2], Angélica Goldoni[1], Fágner Henrique Heldt[3], Daniela Montanari Migliavacca Osório[1], Paulo Günter Windisch[4] and Annette Droste[1,2,5]

Submitted: 30 July, 2012. Accepted: 17 September, 2012

ABSTRACT

Regnellidium diphyllum Lindm. is a heterosporous fern growing in wetlands and humid soils that are being converted to agricultural activities. Many products that are used in agriculture contain copper, resulting in surface and groundwater contamination. Germination and initial development tests were performed using Meyer's solution containing copper sulphate at concentrations of 0 (control), 1, 5, 10, 50 and 100 mg L^{-1}. The experiment was conducted in a growth chamber at 25 ± 1°C for 28 days, with a 12/12-hour light/dark cycle and a photon flux density of 100 µmol m^{-2} s^{-1}. The lowest germination rate (6%) was observed at 100 mg L^{-1}. Primary root growth was significantly reduced at ≥ 10 mg L^{-1}. Secondary leaves of sporophytes grown in concentrations ≥ 5 mg L^{-1} were progressively shorter than were those formed by the control plants. We conclude that the release of pollutants containing copper into the natural habitats of *R. diphyllum* can cause phytotoxicity, threatening the establishment of populations and worsening the already vulnerable conservation status of this species.

Key words: fern, heavy metals, pollution, reproduction, species conservation, wetlands

Introduction

Aquatic ecosystems can be contaminated by heavy metals as a result of natural processes, such as the weathering of rocks, as well as by anthropogenic influences such as industrial activities, agricultural activities and domestic effluents (Soares *et al*. 2000; Hu *et al*. 2010). Because of its widespread use in pesticides and because it is a byproduct of many industrial activities, copper is considered to be a major pollutant (Mal *et al*. 2002). According to the Brazilian National Environmental Council, the current regulatory limit for copper in water is 0.009 mg L^{-1} (Brasil 2005).

As one of the prosthetic groups of enzyme systems and a facultative activator of enzyme systems (Baker 1990), copper is considered to be an essential micronutrient for plants (Arnon & Stout 1939). However, when present in excess, it can be phytotoxic, causing disorders of growth and development by adversely affecting important physiological processes (Yruela 2005). The consequences of copper toxicity are generally more severe for plants in aquatic ecosystems, because they can absorb the metal via roots and leaves, rather than via the roots alone (Guilizzoni 1991).

In the state of Rio Grande do Sul, located in southern Brazil, wetlands are being converted to agricultural uses such as rice cultivation. Approximately 73% of Brazil's 1.3 million hectares under rice cultivation are in Rio Grande do Sul (Primel *et al*. 2005). Rice cultivation contributes to surface and groundwater contamination, because agrochemicals are applied in large quantities to control weeds and to supplement mineral nutrients (FEPAM 2012). Copper accumulation in plants and the consequent phytotoxicity can be attributed to the copper content of the herbicides, fertilizers and fungicides often used in agriculture (Baker 1990, Mal *et al*. 2002, Hu *et al*. 2010). Because plants are known to have different degrees of resistance to heavy metals and to vary in their capacity to accumulate heavy metals (Baker *et al*. 2000), toxic residues of agrochemicals can affect non-target species that naturally occur in wetlands, thereby compromising their conservation status (Terra *et al*. 2008; Droste *et al*. 2010).

Regnellidium diphyllum Lindm. (Marsileaceae) is a heterosporous fern of the Marsileaceae family. Its distribution is restricted to Southern Brazil and some adjoining areas in Uruguay and Argentina (Schultz 1949; Alonso-Paz &

[1] Universidade Feevale, Programa de Pós-Graduação em Qualidade Ambiental, Novo Hamburgo, RS, Brazil
[2] Universidade Feevale, Laboratório de Biotecnologia Vegetal, Novo Hamburgo, RS, Brazil
[3] Pontifícia Universidade Católica de Porto Alegre, Instituto de Pesquisas Biomédicas, Laboratório de Biologia Celular e Molecular, Porto Alegre, RS, Brazil
[4] Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Botânica, Porto Alegre, RS, Brazil
[5] Author for correspondence: annette@feevale.br

Bassagoda 2002). It grows in wetlands, its roots are fixed in humid soil or mud, and its leaves are frequently subjected to flooding (Schultz 1949). The species is currently listed as "vulnerable" on the endangered species list of the state of Rio Grande do Sul (Rio Grande do Sul 2003). Considering the conservation status of this species as well as the continuous destruction and contamination of habitats, studies have been conducted to evaluate the effects of heavy metals and other pollutants on *R. diphyllum* (Wunder *et al*. 2009; Cassanego *et al*. 2010; Droste *et al*. 2010; Kieling-Rubio *et al*. 2010 & 2012).

The objective of this *in vitro* study was to investigate megaspore germination and initial development of *R. diphyllum* sporophytes in the presence of copper, thereby providing information about the impact that copper, as a pollutant, has on the initial stages of the *R. diphyllum* lifecycle.

Material and methods

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

Fifteen sporocarps were washed under tap water, after which they were disinfected with 70% ethanol solution for 30 seconds and 7% sodium hypochlorite solution for 10 minutes before being washed four times in sterile distilled water and dried on sterile filter paper. The sporocarps were then mechanically opened, and megaspores were separated from microspores under stereomicroscopy. Megaspores from different sporocarps were mixed in order to obtain a random sample. Because apogamy can occur naturally in megagametophytes of *R. diphyllum* (Mahlberg & Baldwin 1975), only megaspores were used, the objective being to obtain uniform cultures without mixing sexually and apogamically formed sporophytes. All procedures were conducted under a laminar flow hood.

Meyer's solution was prepared for use as culture medium (Meyer *et al*. 1955) and its pH was adjusted to 5.5 before autoclaving. Copper was added to the culture medium in the form of copper sulphate ($CuSO_4$), at concentrations of 1, 5, 10, 50 and 100 mg L^{-1}. A $CuSO_4$-free culture medium was used as a control solution. Megaspore germination and sporophyte development of *R. diphyllum* in a $CuSO_4$-free medium has previously been described by Wunder *et al*. (2009). Megaspores were kept in glass vials (4.5 × 10 cm) containing 30 mL of Meyer's solution (n = 15 megaspores/vial). Three vials were prepared for each concentration of $CuSO_4$. The experiment was conducted in a growth chamber at 25 ± 1°C, with a 12/12-hour light/dark cycle, under fluorescent lights, which provided a photon flux density of 100 μmol m^{-2} s^{-1}. Megaspores that exhibited at least the initial apical globular green structure with a crown of rhizoids were considered to be germinated (Wunder *et al*. 2009). In order to evaluate sporophytic development, two megaspores were taken at random from each of the vials after 14 and 28 days of cultivation, respectively, resulting in a total of six megaspores for each $CuSO_4$ concentration. The primary root, primary leaf and secondary leaf of each individual were measured. Chlorosis and necrosis of sporophytes were noted and recorded throughout the experiment.

Data were tested for normality using the Shapiro-Wilk test. Germination percentages and mean lengths of roots were compared using the Kruskal-Wallis test followed by the Student-Newman-Keuls test, at a probability of 5%. Mean lengths of primary and secondary leaves were compared using ANOVA followed by Tukey's test, at a probability of 5%. Linear regression analysis was applied to estimate the relationship between $CuSO_4$ concentrations and mean leaf lengths. Statistical analyses were conducted with the Statistical Product and Service Solutions, version 20.0 (SPSS Inc., Chicago, IL, USA) and BioEstat, version 5.0 (Sociedade Civil Mamirauá, Belém, Brazil).

Results

Megaspores germinated at all $CuSO_4$ concentrations. In the $CuSO_4$-free culture medium, 73% of the spores germinated, whereas (at the opposite end of the spectrum) only 6% germinated in the presence of 100 mg L^{-1} of $CuSO_4$ ($H_{5,225}$=12.255, p=0.0315), as shown in Fig. 1.

The majority of germinated megaspores developed into sporophytes with a primary root, a primary leaf with a linear lamina and 1-2 secondary leaves. Sporophyte roots in solutions containing 10, 50 or 100 mg L^{-1} of $CuSO_4$ were significantly shorter than were those in the control medium, after 14 days ($H_{5,30}$=18.1413, p=0.0028) and after 28 days ($H_{5,30}$=23.6838, p<0.001), as can be seen in Fig. 2 (A and B, respectively). From 14 to 28 days, the mean length of the primary root did not increase at 10 or 50 mg L^{-1} of $CuSO_4$ and even decreased at 100 mg L^{-1}.

As shown in Fig. 2C, primary leaf development was unaffected by the presence of copper after 14 days of exposure ($F_{5,30}$=2.663, p=0.051). After 28 days (Fig. 2D), there was a significant negative relationship between $CuSO_4$ concentration and primary leaf length (R^2=0.118, F=5.367, p=0.026) although the difference in relation to the control was significant only at 100 mg L^{-1} ($F_{5,30}$=10.961, p<0.001).

Secondary leaf growth also correlated significantly with $CuSO_4$ concentration after 14 days of exposure (R^2=0.374, F=20.342, p<0.001) and after 28 days of exposure (R^2=0.553, F=42.143, p<0.001). At 14 days (Fig. 2E), secondary leaves of sporophytes grown in 100 mg L^{-1} of $CuSO_4$ were significantly shorter than were those of sporophytes grown in the control solution and in the solutions with lower $CuSO_4$ concentrations ($F_{5,30}$=6.041, p=0.001). At 28 days (Fig. 2F),

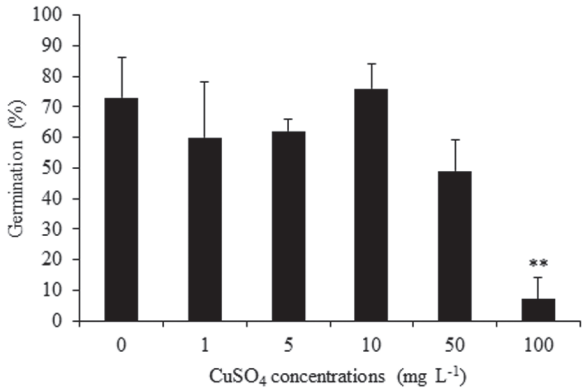

Figure 1. Germination of *Regnellidium diphyllum* Lindm. megaspores in Meyer's solutions containing a range of copper sulphate ($CuSO_4$) concentrations after 28 days. * indicates a significant difference between the treatment and the control ($CuSO_4$-free solution) according to the Student-Newman-Keuls test. Error bars indicate standard deviations.

secondary leaves of sporophytes grown in ≥ 5 mg L^{-1} of $CuSO_4$ were progressively shorter than were those formed of sporophytes grown in the control solution ($F_{5,30}$=14.344, p<0.001). Most of the plants exposed to the highest concentration of copper showed signs of toxicity, such as leaf chlorosis and root necrosis.

Discussion

Copper acts as a structural element in regulatory proteins, as well as participating in photosynthetic electron transport, mitochondrial respiration, oxidative stress responses, cell wall metabolism and hormone signaling (Marschner 1995). Nevertheless, in excess, copper can cause disorders in plant growth and development, affecting key enzymes and altering nitrogen metabolism (Yruela 2005; Soudek *et al*. 2010).

The low proportion of *R. diphyllum* megaspores observed here has also been reported in previous *in vitro* experiments with other metals. Wunder *et al*. (2009) observed that increasing cadmium levels to 50 mg L^{-1} in culture medium resulted in a low *R. diphyllum* germination rate (58%) and that no germination occurred at 100 mg L^{-1}. Kieling-Rubio *et al*. (2010) observed that 50 mg L^{-1} of hexavalent chromium also had a significant negative impact on *R. diphyllum* germination. Those same authors later found that nickel also reduced *R. diphyllum* spore germination, although the germination rate at 100 mg L^{-1} was still 46%, higher than that observed here for the same concentration of copper (Kieling-Rubio *et al*. 2012).

We found that sporophyte development, especially root growth and the development of the secondary leaf, was negatively influenced by excess copper. The decrease in the mean length of the primary root observed at the highest $CuSO_4$ concentration and longest exposure time correlated with senescence and necrosis in root tissue, as previously reported by Kieling-Rubio *et al*. (2012) for *R. diphyllum* exposed to nickel. Roots absorb metals directly from the medium and accumulate those metals (Baker 1990), and such accumulation can therefore cause irreversible damage even in the initial stages of development (Kieling-Rubio *et al*. 2010).

Plants grown in the presence of high levels of copper can exhibit reduced biomass and chlorotic symptoms (Yruela 2005). The consequences of excess copper include reduced chlorophyll content, as well as abnormalities of chloroplast structure and thylakoid membrane composition (Baszynski *et al*. 1988; Quartacci *et al*. 2000). High levels of copper can also affect the uptake of other elements, such as nitrogen, phosphorus, potassium, calcium, magnesium, manganese, zinc and, in particular, iron (Foy *et al*. 1978; Malavolta *et al*. 1997). Interactions between and among elements are often complex and dependent on the plant species involved, the concentrations of elements and the pH of the nutrient solution (Xia & Shen 2007; Koppitke *et al*. 2010).

Sporophytes of *R. diphyllum* are also sensitive to other metals in nutrient solution. Wunder *et al*. (2009) demonstrated that primary root growth and primary leaf growth are significantly reduced and that no secondary leaves develop when *R. diphyllum* is grown in solutions with cadmium concentrations ≥ 12.5 mg L^{-1}. Hexavalent chromium has also been shown to be toxic to *R. diphyllum*, stunting the growth of roots and leaves, at concentrations ≥ 3.2 mg L^{-1} (Kieling-Rubio *et al*. 2010). Kieling-Rubio *et al*. (2012) found that the primary roots, primary leaves and secondary leaves of *R. diphyllum* were all significantly shorter when grown in solutions with nickel concentrations of 3.2 or 4.8 mg L^{-1} than when grown in a nickel-free solution, and that the leaves of plants grown in the 4.8 mg L^{-1} solution exhibited chlorosis and necrosis.

The negative impact of copper on the growth of plantlets, such as that reported here for *R. diphyllum*, has also been observed for other species cultivated in nutrient solutions. Copper concentrations ≥ 3 mg L^{-1} have been shown to cause progressive stunting of growth and reduced CO_2 assimilation in the aquatic fern *Salvinia minima* Baker, a species in which the accumulated concentration of copper was found to be 100 times greater than that normally present in aquatic ecosystems (Al-Hamdani & Blair 2004). Mal *et al*. (2002) demonstrated that *Elodea canadensis* L. C. Rich. in Michx. plants grown for 25 days in nutrient solutions containing 5 or 10 mg L^{-1} of copper exhibited shorter shoots and lower dry mass than did those grown in copper-free solutions. In the angiosperm *Elsholtzia* Willd., hypocotyl and radicle lengths have been shown to be significantly reduced (compared with controls) in plants cultivated for 10 days in solutions containing 50 or 100 µmol L^{-1} of copper (Xia & Shen 2007). Michaud *et al*. (2008) observed severe phytotoxicity symptoms in hydroponic cultures of *Triticum turgidum durum* L. treated with > 1 µmol L^{-1} of copper. However, comparing metal toxicity across studies is often difficult due

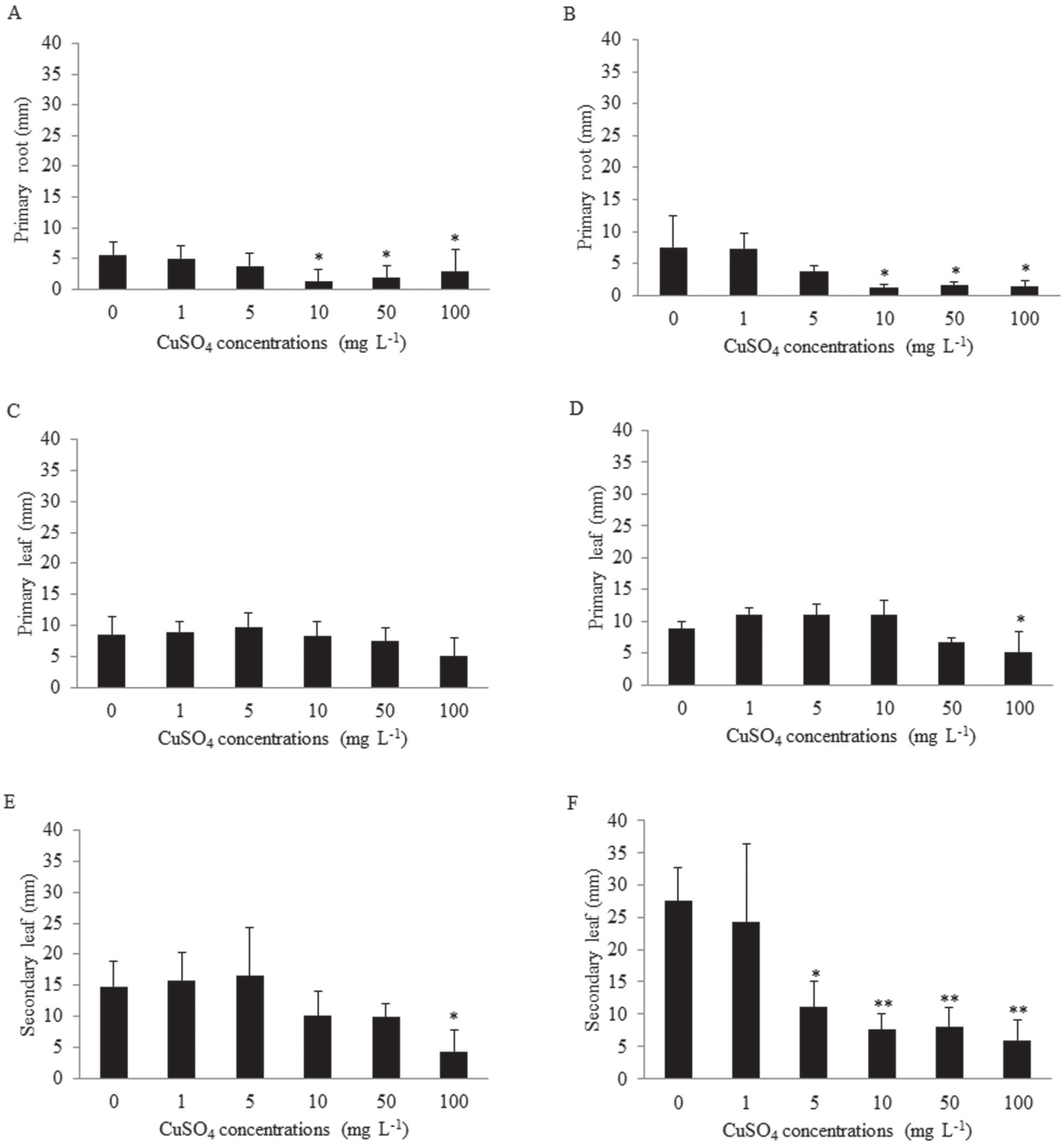

Figure 2. Growth of *Regnellidium diphyllum* Lindm. in Meyer's solutions containing a range of copper sulphate ($CuSO_4$) concentrations. A. Primary root length after 14 days. B. Primary root length after 28 days. C. Primary leaf length after 14 days. D. Primary leaf length after 28 days. E. Secondary leaf length after 14 days. F. Secondary leaf length after 28 days. * indicates a significant difference between the treatment and the control ($CuSO_4$-free solution) according to the Student-Newman-Keuls test (A-B) and Tukey's test (C-F). Error bars indicate standard deviations.

to differences in experimental conditions, which can affect the concentrations that are considered toxic.

The area from which the sporocarps were collected for the present study is used for agricultural activities, primarily rice cultivation (FEPAM 2012). Therefore, it is to be expected that significant quantities of herbicide, fertilizer and fungicide residues would be present in the environment, contributing to copper accumulation and phytotoxicity (Hu *et al*. 2010). Considering the negative impact of copper on *R. diphyllum* germination and sporophyte development, we can posit that contamination of aquatic environments resulting from agricultural activities, especially rice cultivation, poses a risk to the establishment and conservation of natural populations of this already vulnerable species.

Acknowledgements

The authors are grateful to the *Universidade Feevale* for the financial support. Mara Betânia Brizola Cassanego is the recipient of a research grant from the Brazilian *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES, Office for the Advancement of Higher Education).

References

Al-Hamdani, S.H. & Blair, S.L. 2004. Influence of copper on selected physiological responses in *Salvinia minima* and its potential use in copper remediation. **American Fern Journal 94**(1): 47-56.

Alonso-Paz, E. & Bassagoda, M.J. 2002. Revisión de las Marsileaceae del Uruguay y primera cita de *Pilularia americana* A. Braun. **Comunicaciones Botanicas – Museos Nacionales de Historia Natural y Antropologia 125**: 1-8.

Arnon, D.I. & Stout, P.R. 1939. The essentiality of certain elements in minute quantity for plants with special reference to copper. **Plant Physiology 14**: 371-375.

Baker, D.E. 1990. Copper. Pp. 151-175. In Alloway, B.J. (Eds.), **Heavy metals in soils**. London, Chapman & Hall.

Baker, A.J.M.; McGrath, S.P.; Reeves, R.D. & Smith, J.A.C. 2000. Metal hyperaccumulator plants: a review of the ecology and physiology of a biochemical resource for phytoremediation of metal polluted soils. Pp. 85-107. In Terry, N.; Banuelos, G. & Vangrnsveld, J. (Eds.), **Phytoremediation of contaminated soil and water**. Boca Raton, Lewis.

Baszynski, T.; Tukendorf, A.; Ruszkowska, M.; Skórzynska, E. & Maksymiec, W. 1988. Characteristics of the photosynthetic apparatus of copper non-tolerant spinach exposed to excess copper. **Journal of Plant Physiology 132**: 708-713.

Brasil. 2005. Resolução CONAMA no. 357, de 17 de março de 2005. Dispõe sobre a classificação dos corpos de água e diretrizes ambientais para o seu enquadramento, bem como estabelece as condições e padrões de lançamento de efluentes, e dá outras providências. **Diário Oficial da União**, Poder Executivo, Brasília, 18 de março de 2005.

Cassanego, M.B.B.; Droste, A. & Windisch, P.G. 2010. Effects of 2,4-D on the germination of megaspores and initial development of *Regnellidium diphyllum* Lindman (Monilophyta, Marsileaceae). **Brazilian Journal of Biology 70**(2): 361-366.

Droste, A.; Cassanego, M.B.B. & Windisch, P.G. 2010. Germination and sporophytic development of *Regnellidium diphyllum* Lindm. (Marsileaceae) in the presence of a glyphosate-based herbicide. **Revista Brasileira de Biociências 8**(2): 174-178.

Foy, C.D.; Chaney, R.L. & White, M.C. 1978. The physiology of metal toxicity in plants. Pp. 511-566. In: **Annual Review Plant Physiology 29**.

Fundação Estadual de Proteção Ambiental Henrique Luis Roessler - FEPAM. Available at: <http://www.fepam.rs.gov.br>. (Accesso em 24/06/2012).

Guilizzoni, P. 1991. The role of heavy metals and toxic materials in the physiological ecology of submersed macrophytes. **Aquatic Botany 41**: 87-109.

Hu, J.; Zheng, A.; Pei, D. & Shi, G. 2010. Bioaccumulation and chemical forms of cadmium, copper and lead in aquatic plants. **Brazilian Archives of Biology and Technology 53**(1): 235-240.

Kieling-Rubio, M.A.; Droste, A. & Windisch, P.G. 2010. Germination and sporophytic development of *Regnellidium diphyllum* Lindman (Marsileaceae) in the presence of hexavalent chromium. **Brazilian Journal of Biology 70**(4): 1149-1153.

Kieling-Rubio, M.A.; Droste, A. & Windisch, P.G. 2012. Effects of nickel on the fern *Regnellidium diphyllum* Lindm. (Marsileaceae). **Brazilian Journal of Biology 72**(4): in press.

Koppitke, P.M.; Blamey, F.P.C.; Asher C.J. & Menzies, N.W. 2010. Trace metal phytotoxicity in solution culture: a review. **Journal of Experimental Botany 61**(4): 945-954.

Mahlberg, P.G. & Baldwin, M. 1975. Experimental studies on megaspore viability, partenogenesis and sporophyte formation in *Marsilea*, *Pilularia* and *Regnellidium*. **Botanical Gazette 136**: 269-273.

Mal, T.K.; Adorjan, P. & Corbett, A.L. 2002. Effect of copper on growth of an aquatic macrophyte, *Elodea canadensis*. **Environmental Pollution 120**: 307-311.

Malavolta, E.; Vitti, G.C. & Oliveira, S.A. 1997. **Avaliação do estado nutricional das plantas: princípios e aplicações**. 2 ed. Piracicaba, Potafos.

Marschner, H. 1995. **Mineral nutrition of higher plants**. 2 ed. London, Academic Press.

Meyer, B.S.; Anderson, D.B. & Swanson, C.A. 1955. **Laboratory Plant Physiology**. New York, Van Nostrand.

Michaud, A.M.; Chappellaz, C. & Hinsinger, P. 2008. Copper phytotoxicity affects root elongation and iron nutrition in durum wheat (*Triticum turgidum durum* L.). **Plant Soil 310**: 151-165.

Primel, E.G.; Zanella, R.; Kurz, M.H.S.; Gonçalves, F.F.; Machado, S.O. & Marchezan, E. 2005. Poluição das águas por herbicidas utilizados no cultivo do arroz irrigado na região central do estado do Rio Grande do Sul, Brasil: predição teórica e monitoramento. **Química Nova 28**(4): 605-609.

Quartacci, M.F.; Pinzino, C.; Sgherri, C.L.M.; Dalla Vecchia, F. & Navari-Izzo, F. 2000. Growth in excess copper induces changes in the lipid composition and fluidity of PSII-enriched membranes in wheat. **Physiologia Plantarum 108**: 87-93.

Rio Grande do Sul. 2003. Decreto n° 42.099, de 31 de dezembro 2002. Declara as espécies da flora nativa ameaçadas de extinção do Estado do Rio Grande do Sul e dá outras providências. Pp. 1-6. **Lex-Diário Oficial do Rio Grande do Sul**, ano LXII, nº1.

Schultz, A.R. 1949. Contribuições ao conhecimento de *Regnellidium diphyllum* Lindman. **Lilloa XVII**: 139-144.

Soares, C.R.F.S.; Siqueira, J.O.; Carvalho, J.G.; Moreira, F.M.S. & Grazziotti, P.H. 2000. Crescimento e nutrição mineral de *Eucalyptus maculata* e *Eucalyptus urophylla* em solução nutritiva com concentração crescente de cobre. **Revista Brasileira de Fisiologia Vegetal 12**(3): 213-225.

Soudek, P.; Katrusáková, A.; Sedlácek, L.; Petrová, S.; Kocí, V.; Marsik. P.; Griga, M. & Vanek, T. 2010. Effect of heavy metals on inhibition of root elongation in 23 cultivars of flax (*Linum usitatissimum* L.). **Archives of Environmental Contamination and Toxicology 59**: 194-203.

Terra, N.R.; Feiden, I.R.; Fachel, J.M.G.; Lemos, C.T. & Nunes, E.A. 2008. Ecotoxicological evaluation of sediment and water samples from Sinos River, Rio Grande do Sul, Brazil, using *Daphnia magna* and V79 cells. **Acta Limnologica Brasilica 20**(1): 63-72.

Xia Y. & Shen Z.G. 2007. Comparative studies of copper tolerance and uptake by three plant species of the genus *Elsholtzia*. **Bulletin of Environmental Contamination and Toxicology 79**: 53-57.

Wunder, D.A.; Droste, A. & Windisch, P.G. 2009. Megaspore germination and initial development of *Regnellidium diphyllum* Lindman (Pteridophyta, Marsileaceae) sporophytes in the presence of cadmium. **Revista Brasileira de Botânica 31**: 177-181.

Yruela, I. Copper in plants. 2005. **Brazilian Journal of Plant Physiology 17**(1): 145-156.

Online version: www.scielo.br/abb and http://www.botanica.org.br/acta/ojs