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Germination and sporophytic development of *Regnellidium diphyllum* Lindm. (Marsileaceae) in the presence of a glyphosate-based herbicide

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ABSTRACT: (Germination and sporophytic development of *Regnellidium diphyllum* Lindm. (Marsileaceae) in the presence of a glyphosate-based herbicide). *Regnellidium diphyllum* is a vulnerable heterosporous fern which occurs in the State of Rio Grande do Sul, Brazil, and in some neighboring localities in the State of Santa Catarina, in Uruguay and Argentina. The species grows in areas subjected to flooding and humid soils which frequently are altered by agricultural activities. Agricultural fields are commonly treated with herbicides such as glyphosate. The effects of glyphosate on *in vitro* germination of megaspores and initial sporophytic development of *R. diphyllum* under aqueous conditions were investigated. Six glyphosate concentrations (0.32, 0.64, 1.92, 4.80, 9.60 and 19.20 mg/L) and the control (0.00 mg/L) were tested using Meyer's medium. Cultures were maintained *in vitro* in a growth chamber at 24±1°C and 16 hours photoperiod for five weeks. Megaspore germination was significantly reduced (67% and lower) in concentrations of 4.80 mg/L onwards compared with the control (81%), while the sporophyte formation was negatively influenced even at the lowest concentration tested (0.32 mg/L). The length of the primary root and primary and secondary leaves was significantly reduced at glyphosate concentrations of 4.80 mg/L onwards. Low concentrations of the herbicide did not affect the percentage of germination, although significantly interfered in the initial development of *R. diphyllum*. Concentrations of 9.60 and 19.20 mg/L inhibited the establishment of new plants. These results indicate that application of glyphosate to agricultural areas may have negative impacts on the vulnerable fern *R. diphyllum*.

Key words: heterosporous fern, ecophysiology, pollution, reproduction, conservation.

RESUMO: (Germinação e desenvolvimento esporofítico de *Regnellidium diphyllum* Lindm. (Marsileaceae) na presença de um herbicida baseado em glifosato). *Regnellidium diphyllum* é uma pteridófito heterosporada vulnerável, ocorrente no estado do Rio Grande do Sul, Brasil e em algumas localidades vizinhas no estado de Santa Catarina, no Uruguai e na Argentina. A espécie cresce em áreas alagáveis e solos úmidos que frequentemente são alterados para atividades agrícolas. Campos de agricultura são comumente tratados com herbicidas como o glifosato. Os efeitos do glifosato sobre a germinação de megásporos e o desenvolvimento esporofítico inicial de *R. diphyllum* foram investigados. Seis concentrações de glifosato (0,32; 0,64; 1,92; 4,80; 9,60 e 19,20 mg/L) e o controle (0,00 mg/L), foram testados utilizando meio de Meyer. As culturas foram mantidas *in vitro*, em câmara de germinação a 24±1°C e fotoperíodo de 16 horas, por cinco semanas. A germinação de megásporos foi significativamente reduzida (67% e inferior) em concentrações de glifosato a partir de 4,80 mg/L, comparada ao controle (81%), enquanto que a formação de esporófitos foi negativamente influenciada mesmo na menor concentração testada (0,32 mg/L). Os comprimentos da raiz primária e das folhas primária e secundária foram significativamente reduzidos em concentrações de glifosato a partir de 4,80 mg/L. Baixas concentrações do herbicida não afetaram a porcentagem de germinação, embora tenham interferido significativamente no desenvolvimento inicial de *R. diphyllum*. Concentrações de 9,60 e 19,20 mg/L inibiram o estabelecimento de plantas novas. Esses resultados indicam que a aplicação de glifosato em áreas de agricultura pode ter impactos negativos sobre a pteridófito vulnerável *R. diphyllum*.

Palavras-chave: filicínea heterosporada, ecofisiologia, poluição, reprodução, conservação.

INTRODUCTION

Wetlands are increasingly being altered for agricultural activities and grazing. In Brazil, rice fields have been established extensively in flat areas of the state of Rio Grande do Sul, including coastal plains. Rice cultivation is considered as an activity with high potential for pollution (Fepam 2009), as agrochemicals are intensely used to control weeds and to increment crop yield. Although weed control using large spectrum herbicides has become effective (Usui 2001), the excessive or improper application of these products can cause contamination of surface

and ground water (Cerejeira *et al.* 2003, Marchesan *et al.* 2007). The exposure of non-target organisms in environments contaminated by herbicide spraying, lateral drift and runoff is a major concern for the conservation of native animal and plant species, justifying ecotoxicological studies (Coler *et al.* 2005, Luo & Ikeda 2007).

Glyphosate (N-(phosphonomethyl) glycine) is a large spectrum herbicide commonly used for the control of monocotyledonous and dycotyledonous weeds in terrestrial and aquatic environments, especially in shallow water systems (Tsui & Shu 2003). It is the most widely used non-selective systemic herbicide worldwide, representing

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60% of the market of non-selective herbicides (Amarante Jr. & Santos 2002). In Brazil, this herbicide is not included in the National Environmental Council (CONAMA) listing that establishes the maximal levels of metals and herbicides allowed in water and soil (MMA 2009).

In soil, glyphosate is adsorbed by clay and organic colloids and its degradation occurs by microbial action, so that it has no long term residual action, whereas, in aquatic systems, its degradation is slow (Hertwig 1983). The absorption of glyphosate by plants occurs through leaves and young caulinar axes with subsequent translocation to all tissues. Glyphosate interacts with many enzymatic systems throughout the plant, interfering in the formation of aromatic amino acids (Ralph 2000). The sensitivity and vulnerability to herbicides of higher plants in aquatic systems have been studied (Keary *et al.* 2000, Santos & Banzatto 2000, Sheffield 2002, Aida *et al.* 2006, Luo & Ikeda 2007).

Regnellidium diphyllum Lindm. is a heterosporous fern and belongs to the family Marsileaceae. Its distribution is restricted to Southern Brazil and some adjoining localities in Uruguay and Argentina (Schultz 1949, Alonso-Paz & Bassagoda 2002). It grows in wetlands and the rhizome develops in the humid soil or mud. The plants are frequently subjected to flooding. Currently, this species is on endangered species list of the State of Rio Grande do Sul, Brazil (Sema 2009). Considering the conservation status of *R. diphyllum* and the lack of information about its sensitivity to pollutants, germination of megaspores and initial development of sporophytes in the presence of glyphosate were investigated *in vitro*, providing information on the influence of the herbicide in the establishment and initial growth of this species.

MATERIAL AND METHODS

Mature sporocarps of *Regnellidium diphyllum* were obtained from different plants in the Triunfo country (State of Rio Grande do Sul, Brazil; 29°48'S, 51°41'W). Sporocarps were kept at room temperature (about 25°C) for 30 days until the experiment onset. Voucher specimens were deposited at the Herbarium Anchieta (PACA), Universidade do Vale do Rio dos Sinos, São Leopoldo, Brazil.

In a flow chamber, sporocarps were washed under tap water, rinsed with 70% ethanol solution for 30 seconds, kept for ten minutes in a sodium hypochlorite solution (7%, v/v), rinsed four times in sterile distilled water and blotted on sterile filter paper at room temperature. Fifteen sporocarps were mechanically cracked, liberating the spores, and the megaspores were manually separated from the microspores under a stereo-microscope. To assure homogeneous megaspore samples, the materials from all sporocarps were mixed. As apogamy normally occurs in megagametophytes of *R. diphyllum* (Mahlberg & Baldwin 1975), only megaspores were used in order to obtain uniform cultures and avoid the formation of a mixture of sexual and apogamically-formed sporophytes.

Glyphosate was tested using the commercial formulation of Glifos® (480 g/L active ingredient (i.e. glyphosate; Cheminova Brasil Ltda.). Manufacturer recommendations indicate the application of 2 L per hectare of cultivated rice (960 g/hectare). Considering the occasional submersion of *R. diphyllum* plants in water up to ca. 30 cm it would correspond to a cover of three million liters of water per hectare, resulting in the dilution of the applied glyphosate to a concentration of 0.32 mg/L. Higher concentrations tested would represent herbicide applications over shallower water bodies, additional input through other means or accidental drift over humid soil.

Meyer's solutions (Meyer *et al.* 1955) were prepared as culture media and the pH was adjusted to 6.0 before autoclaving. Different concentrations of glyphosate from commercial formulation were sterilized through micro-filtration (microporous filter Millipore™) added to the sterile media: 0.00 (control), 0.32, 0.64, 1.92, 4.80, 9.60 and 19.20 mg/L. Twenty-five megaspores were placed in each glass vial (4.5 cm x 10 cm) with 25 mL of the solution and six replicates for each treatment were used. Cultures were maintained in a growth chamber at 24±1°C, under artificial light intensity of 110 µmol m⁻²s⁻¹ and 16-hour photoperiod.

In order to record the development of the sporophytes, one individual was randomly taken from each replicate, in a laminar flux chamber, after 7 and 35 days of exposure to glyphosate. The length of the primary root and the length of the primary and secondary leaves were measured, plants were photographed, and then fixed in ethanol (70%, v/v).

Germinated megaspores and gametophytes presenting sporophytes were counted at day 35. For each treatment, the total number of leaves was counted for one individual from each replicate, totalizing six individuals per treatment. Megaspores presenting at least the initial apical globular green structure with a crown of rhizoids were considered as germinated. Morphological details of sporophyte development under different glyphosate concentrations were recorded.

Megaspore germination and sporophyte formation numbers were transformed into percentages. The normality and homogeneity of the data were verified using the Wilk-Shapiro and the Levene tests. The One-Way ANOVA test was applied to the data that showed normality and homogeneity. The difference between means was verified using the Tukey test with p<0.05. The Kruskal-Wallis test was applied to data that did not show normality and homogeneity and differences between means were verified using the Dunn test with significance level of 5% (Zar 1999). Linear regression analysis was applied to estimate the relation between the concentrations of glyphosate and the respective number of leaves formed.

RESULTS AND DISCUSSION

From a total of 1050 megaspores of *Regnellidium diphyllum* used in the experiment, 744 germinated (71%)

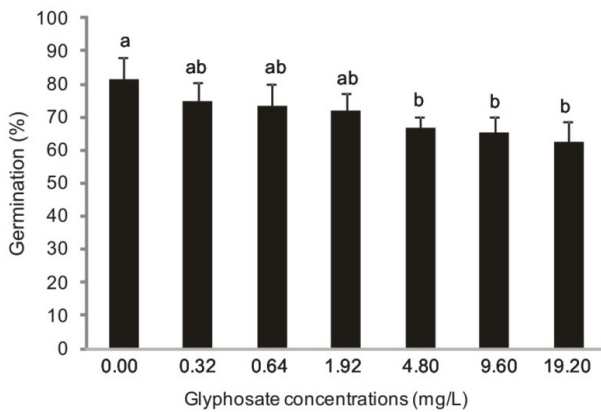


Figure 1. Germinability of *Regnellidium diphyllum* Lindman megaspores in different glyphosate concentrations after 35 days of exposure (ANOVA test, $F=7.94$, $d.f.=6, 35$, $p<0.001$). Means with different letters are significantly different by the Tukey test, $p<0.05$. Error bars indicate standard deviation.

and 511 (49%) formed sporophytes. A significant negative influence of glyphosate in the germination of megaspores and the formation of sporophytes was observed. Glyphosate concentrations of 4.80 mg/L onwards reduced significantly the germination percentage in relation to the control (Fig. 1). Although glyphosate is known to be less effective on seeds (Amarante Jr. & Santos 2002), excessive applications or high doses of this herbicide may inhibit the germination process of some species (Rodrigues & Almeida 1998).

With exception of the lowest glyphosate concentration tested, all treatments presented significant differences in the percentage of gametophytes with sporophytes when compared to the control (Fig. 2). In concentrations of 4.80 mg/L onwards, sporophyte percentages were lower than 60%, demonstrating the negative effect of the herbicide.

After one week in culture, growth of sporophytic structures in the control treatment was about 3 and 5 mm for roots and primary leaves, respectively. Similar results were observed by Mahlberg & Baldwin (1975). In our experiments, glyphosate affected the length of primary roots as well as primary and secondary leaves of the sporophytes (Tab. 1). After seven days of exposure,

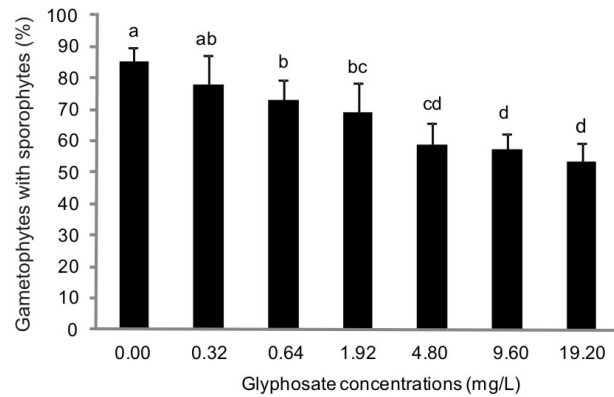


Figure 2. Formation of sporophyte in *Regnellidium diphyllum* Lindman under different glyphosate concentrations after 35 days of exposure (Kruskal-Wallis test, $H=29.28$, $d.f.=6, 35$, $p<0.001$). Means with different letters are significantly different by the Dunn test, $p<0.05$. Error bars indicate standard deviation.

a significant reduction in root growth compared to the control was observed at concentrations of 1.92 mg/L onwards. After 35 days, these differences could still be observed in concentrations of 0.64 mg/L onwards.

After seven days, the growth of the primary leaf was significantly reduced at glyphosate concentrations of 1.92 mg/L and higher when compared with the control. After 35 days, the length of the primary leaf was significantly affected in 9.60 mg/L and higher concentrations (Tab. 1).

The development of the secondary leaf was strongly affected by glyphosate after seven and 35 days of exposure (Tab. 1). This structure is usually formed after one week in culture (Vianna 1973), but in the treatments with 9.60 and 19.20 mg/L of glyphosate, no secondary leaf was formed after seven days of exposure. Although all glyphosate concentrations tested permitted the formation of secondary leaves after 35 days of exposure, leaves were significantly shorter in the glyphosate treatments than in the control.

Morphological abnormalities could be observed at the end of 35 days of exposure of plantlets to glyphosate. Sporophytes were smaller and presented necrotic tissues at the proximal end of petioles as well as chlorotic areas

Table 1. *Regnellidium diphyllum* Lindman growth of primary root, primary leaf and secondary leaf after seven and 35 days of exposure at different glyphosate concentrations (mean \pm standard deviation (SD)). Different letters in the column indicate significant differences among treatments by the Dunn test ($p<0.05$).

Glyphosate (mg/L)	Primary root (mm)		Primary leaf (mm)		Secondary leaf (mm)	
	mean \pm SD		mean \pm SD		mean \pm SD	
	7 days	35 days	7 days	35 days	7 days	35 days
0.00	4.7 \pm 1.2 a	7.5 \pm 1.0 a	6.3 \pm 0.8 a	10.0 \pm 1.1 ab	2.8 \pm 1.2 a	18.3 \pm 2.2 a
0.32	4.3 \pm 1.2 a	6.8 \pm 0.7 a	5.3 \pm 0.5 a	10.7 \pm 1.0 a	2.3 \pm 1.2 a	15.8 \pm 1.5 b
0.64	3.7 \pm 1.6 ab	5.5 \pm 0.8 b	5.0 \pm 1.4 ab	9.3 \pm 1.0 ab	1.7 \pm 0.5 a	14.7 \pm 3.1 b
1.92	2.5 \pm 0.5 b	5.3 \pm 1.0 b	4.0 \pm 0.6 bc	9.2 \pm 0.7 ab	0.8 \pm 0.2 b	14.0 \pm 0.9 b
4.80	2.7 \pm 1.3 bc	4.8 \pm 1.2 b	4.2 \pm 0.7 bc	8.8 \pm 0.7 b	0.7 \pm 0.2 b	11.2 \pm 1.7 c
9.60	1.5 \pm 0.5 c	2.8 \pm 0.7 c	3.2 \pm 0.7 c	4.8 \pm 1.2 c	0.0 c	6.0 \pm 1.7 d
19.20	0.7 \pm 0.2 d	1.3 \pm 0.5 d	0.7 \pm 0.2 d	1.2 \pm 0.4 d	0.0 c	1.7 \pm 0.5 e
	H=29.60	H=34.15	H=31.86	H=30.21	H=36.17	H=35.68
	df= 6, 35	df= 6, 35	df= 6, 35	df= 6, 35	df= 6, 35	df= 6, 35
	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001

on leaf lamina. These alterations were more obvious in treatments of 1.92 mg/L of herbicide onwards. Most of the sporophytes exposed to 19.20 mg/L of glyphosate died after 35 days of exposure.

Linear regression analysis revealed a significant negative relation between the glyphosate concentrations and the number of leaves formed after 35 days of exposure (Fig. 3). In the control and in 0.32 mg/L of the herbicide, the sporophytes formed an average of five leaves, whereas concentrations of 0.64 mg/L onwards caused a gradual decrease of the maximum number of leaves formed (declining from five to two). The mean number of leaves formed in each treatment corresponded closely to the regression line (Fig. 3). Specially, at the highest glyphosate concentration (19.20 mg/L), the plantlets showed the lowest numbers of leaves (average of 1.5).

The strong effect of glyphosate on juvenile plants is related to the fact that the herbicide acts directly on the photosynthetic parts, specially the leaves (Hertwig 1983). Glyphosate rapidly crosses the cuticle and the cell wall, reaching the interior of cells. As a systemic herbicide, it is translocated throughout the entire plant, causing biochemical disturbance in cells by inhibiting the 5-enolpyruvylshikimate-3-phosphate-synthase enzyme (EPSPS). Therefore, it interferes in the metabolic pathway of the synthesis of essential aromatic amino acids (phenylalanine, tyrosine and tryptophan), precursor molecules of compounds such as lignin, alkaloids, flavonoids and benzoic acid that interact in the plant metabolic processes like transpiration, respiration and ethylene production (Hertwig 1983, Amarante Jr. & Santos 2002).

Investigations have focused on the sensitivity of ferns to rice herbicides (Sheffield 2002, Aida *et al.* 2006, Luo & Ikeda 2007). Herbicides can widely vary in the mode of action and in consequent effects across various phylogenetic groups of plants (Fairchild *et al.* 1997), due to their different capacities to transform and degrade herbicides, since different types and activities of enzymes work on this metabolism (Usui 2001). Few studies have evaluated

the effects of glyphosate on ferns (Nelson *et al.* 2001, Fairchild *et al.* 2002) and no tests have been performed on the toxicity of this herbicide on the Marsileaceae family, what makes inter-family comparisons difficult. Wong (2000) tested concentrations of 0.02 to 200 mg/L of the herbicide 2,4-D, glyphosate and paraquat on the growth and chlorophyll-*a* synthesis of the green alga *Scenedesmus quadricauda* Berb 614. In this experiment he found that the presence of 2 mg/L of the herbicide *in vitro* significantly inhibited the algal growth and the photosynthetic rate to about 60% of the control, while the presence of 20 mg/L or more impede the growth totally. Glyphosate did not significantly affect the photosynthetic capacity of the seagrass *Halophila ovalis*, even at concentrations 100-fold higher than for other herbicides tested (Ralph 2000). Santos *et al.* (2001) verified the effect of sublethal doses of herbicides added to nutrient solutions commonly used to cultivate *Spirodela punctata* (Lemnaceae). Glyphosate was less toxic than the other herbicides tested, inhibiting the multiplication rate from 35 mg/L upwards. After seven days of *in vitro* culture, a positive relation between glyphosate concentration and frond death was observed.

The efficacy of glyphosate against the giant salvinia (*Salvinia molesta*), an invasive aquatic heterosporous fern was demonstrated in two studies (Nelson *et al.* 2001, Fairchild *et al.* 2002). Santos & Banzatto (2000) analyzed the effects of different concentrations of glyphosate (6.25, 12.5, 25.0 and 50.0 mg/L) on two macrophyte species, including *Salvinia minima*. They found that the species is sensitive to the herbicide, since multiplication of fronds was drastically decreased with 50 mg/L, while in the present study with *Regnellidium diphyllum* at 19.20 mg/L most sporophytes were morphologically abnormal and died by the end of the 35th day of exposure, indicating that this fern is more sensible to this herbicide than *S. minima*. Species of *Salvinia*, mainly *S. minima* and *S. molesta*, are frequently found in environments also preferred by *Regnellidium diphyllum*.

In Brazil, glyphosate is not included in the National Environmental Council (CONAMA) listing that establishes the maximal levels of metals and herbicides allowed in water and soil (MMA 2009). Although glyphosate may be considered to be non toxic to plants in low concentrations, the lack of long-term studies does not allow for conclusions as to deleterious effects after long time use of the commercial products (Tsui & Chu 2003). In the present study, the growth of *Regnellidium diphyllum* sporophytes was diminished in the glyphosate concentration recommended for agricultural use (0.32 mg/L). The constant use of glyphosate, mainly in the crop fields created in river plains and close to wetlands may interfere in the establishment of *R. diphyllum* and has to be considered as an additional threat to this species. Therefore, further studies of the effects of glyphosate on *R. diphyllum* and other aquatic, semi-aquatic, and terrestrial ferns are needed.

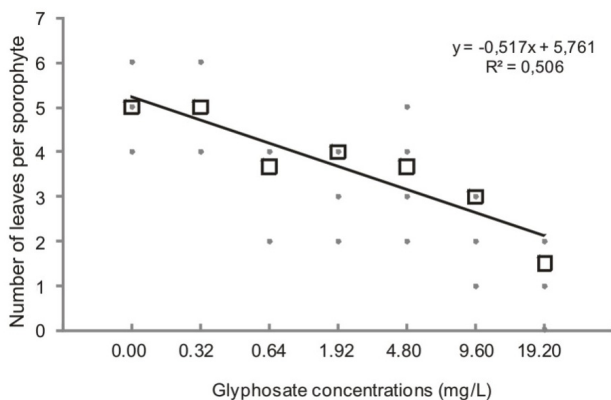


Figure 3. Relation between the number of leaves of *Regnellidium diphyllum* Lindman sporophytes and different glyphosate concentrations after 35 days of exposure (ANOVA test, $F=42.85$, $d.f.=1, 40$, $p<0.001$). Grey spheres represent one or more of the six replicates; white squares represent the mean number of leaves in each treatment.

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