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Article

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Acute Toxicity of Herbicides and Sensibility of Aquatic Plant *Wolffia brasiliensis* as a Bioindicator Organism

Toxicidade Aguda de Herbicidas e Sensibilidade da Planta Aquática ***Wolffia brasiliensis*** *como Organismo Bioindicador*

ABSTRACT - This study aimed to establish the mass cultivation of *Wolffia brasiliensis* under laboratory conditions, determine the sensitivity of reference substances (potassium dichromate, sodium chloride, and potassium chloride), and estimate the acute toxicity (LC50;7d) for the herbicide glyphosate, diquat, paraquat, imazapyr, 2,4-D, saflufenacil, imazamox, penoxsulam, metsulfuron-methyl, and atrazine. Three media were tested for culture establishment: Hoagland's medium, 5.0 g L^{-1} NPK fertilizer (20-5-20), a mixture of Oxisol + organic substrate of decaying aquatic plant (2:1; v v^{-1}). Sensitivity tests with reference substances and acute toxicity for herbicides were carried out after establishing the cultivation. The species *W. brasiliensis* presented an excellent sensitivity response to reference substances, with means of LC50;7d of 15.61±2.13 g L^{-1}, 17.02±0.50 g L^{-1}, and 52.54±4.27 mg L^{-1} for sodium chloride, potassium chloride, and potassium dichromate, respectively. The acute toxicity response indicated that *W. brasiliensis* was extremely sensitive to diquat (LC50;7d = 0.07 mg L^{-1}) and paraquat (LC50;7d < 0.05 mg L^{-1}), sensitive to imazapyr (LC50;7d = 21.10 mg L^{-1}), and little sensitive to the other herbicides. The response capacity for the different groups of herbicides, the standardized response to reference substances, and ease of cultivation in the laboratory indicate the potential for using this aquatic plant in the assessment of herbicide risk in aquatic environments.

Keywords: macrophytes, chemicals, ecotoxicology, reference substances, pesticides.

RESUMO - Os objetivos deste estudo foram estabelecer o cultivo massal de ***W. brasiliensis*** *em condição de laboratório, determinar a sensibilidade para as substâncias-referência (dicromato de potássio, cloreto de sódio e cloreto de potássio) e a estimar a toxicidade aguda (CL50;7d) para os herbicidas glyphosate, diquat, paraquat, imazapyr, 2,4-D, saflufenacil, imazamox, penoxsulam, metsulfuron-methyl e atrazine. Para estabelecimento do cultivo, foram testados três meios: Hoagland's; 5,0 g L^{-1} de fertilizante NPK (20;5;20) e o da mistura Latossolo Vermelho + substrato orgânico de planta aquática em decomposição (2:1; v v^{-1}). Após o estabelecimento do cultivo, foram realizados os ensaios de sensibilidade com as substâncias-referência e os ensaios de toxicidade aguda para os herbicidas. A* ***W. brasiliensis*** *apresentou excelente resposta de sensibilidade às substâncias-referência com CL50;7d média de 15,61±2,13 g L^{-1} para o cloreto de sódio, de 17,02±0,50 g L^{-1} para o cloreto de potássio e de 52,54±4,27 mg L^{-1} para o dicromato de potássio. A resposta de toxicidade aguda de W. brasiliensis para o diquat (CL50;7d = 0,07 mg L^{-1}) e paraquat (< 0,05 mg L^{-1}) indica que a planta pode ser considerada extremamente sensível; para o imazapyr, sensível (CL50;7d = 21,10 mg L^{-1}), e para os demais herbicidas testados, pouco sensível. A capacidade de resposta relativa a diferentes grupos de herbicidas somada à resposta*

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padronizada às substâncias-referência e facilidade de cultivo em laboratório indicam potencial para o uso desta planta aquática na avaliação de risco de herbicidas em ambientes aquáticos.

Palavras-chave: macrófitas, produtos químicos, ecotoxicologia, substâncias-referência, agrotóxicos.

INTRODUCTION

Pollution caused by agriculture has caused severe problems in several countries due to the excessive use of fertilizers, herbicides, insecticides, and other chemicals during the production cycles. Water pollution by agricultural activity is a critical problem (Chen et al., 2017). Aquatic environments are hit by complex mixtures of chemicals, which may represent risks to ecosystems and human health (Brack et al., 2016).

In this context, environmental monitoring has become a prominent tool (Brack et al., 2016), but a problem of chemical monitoring is the knowledge of the risk that each substance may represent, its carcinogenic and mutagenic properties, toxicity, potential bioaccumulation or persistence, hindering the real establishment of risk of environmental exposure (Johnson et al., 2017). Toxicity tests with bioindicator organisms are essential during the entire environmental assessment process as they incorporate the interaction between contaminants and environmental properties and allow the ecological assessment of effects derived from exposure (Masese, 2013). Thus, biomonitoring can be performed with organisms representing different ecological groups, such as algae, aquatic plants, aquatic invertebrates, benthic organisms, macroinvertebrates, fishes, and periphytic communities (Laini et al., 2018).

Studies using aquatic plants as bioindicators of exposure or toxicity of chemicals have been carried out with *Lemna minor* for copper sources and herbicide diquat (Garlich et al., 2016) and pharmaceuticals and personal care products (PPCPs) (Ebele et al., 2017); *Azolla caroliniana* for herbicides (Silva et al., 2012; Cruz et al., 2015); *Pistia stratiotes*, *Eichhornia* spp., *Lemna* spp., and *Salvinia* spp. for heavy metals (Rezania et al., 2016); and *L. minor* and *A. caroliniana* for herbicides, fungicides, and insecticides (Della Vechia et al., 2016), among others.

The development of new bioindicator organisms is important to increase the representativeness of assessed ecological groups. This type of study requires a series of assessments on its adaptation to the cultivation under laboratory conditions, validation of response to the reference substance (Cruz et al., 2008), behavior during the toxicity test, visibility of the concentration-response relationship, and quantification and qualification of response.

Therefore, the aquatic plant *Wolffia brasiliensis* can be assessed as a bioindicator organism in environmental monitoring and ecotoxicology studies. This plant belongs to the family Lemnaceae (monocot), have free-floating fronds on the water surface, globose, ovoid to subcircular, with distribution in tropical-subtropical and warm-temperate regions of Americas (Dahlgren et al., 1985). *W. brasiliensis* has been used to assess the toxicity of zinc sulfate (Lahive et al., 2011) and *W. globosa* for silver nanoparticles (AgNPs) (Zou et al., 2017). Due to the scarcity of bioindicator aquatic plants in the literature, especially in tropical environments, this study aimed to assess the establishment of *W. brasiliensis* cultivation under laboratory conditions, determine the sensitivity for reference substances (potassium dichromate, sodium chloride, potassium chloride), and estimate acute toxicity (LC50;7d) for the herbicides 2,4-D, atrazine, diquat, glyphosate, imazapyr, imazamox, metsulfuron-methyl, penoxsulam, paraquat, and saflufenacil.

MATERIAL AND METHODS

Collection and establishment of mass cultivation

The species *W. brasiliensis* was collected in lagoons and dams with the following geographical coordinates: S 20°35'16.66" and W 48°32'21.24". Then, part of the sample was transferred to the laboratory condition; one part was maintained in a greenhouse, and the other under a microcosm condition.

Three culture media were tested for the establishment of *W. brasiliensis* under laboratory conditions: Hoagland's, as recommended for *Lemna* sp. (OECD, 2002); 5.0 g L^{-1} of NPK fertilizer (20-5-20); and 1.5 kg L^{-1} of the mixture Oxisol + organic substrate of decaying aquatic plant (2:1; v v^{-1}). Plastic black boxes with a capacity of 2.5 L were used in the laboratory for each type of test medium. This culture test was performed at a temperature between 25.0 and 27.0 °C, lighting of 1,000 lux, and 12 hour light/dark photoperiod.

These culture media were also tested under greenhouse conditions at temperatures between 27.0 and 30.0 °C. The mixture of 10 kg of Oxisol + organic substrate and the medium with 5.0 g L^{-1} of fertilizer (NPK) were tested in 200 L microcosms under natural environment.

Tests of sensitivity to reference substances

Reference substances consisted of sodium chloride (NaCl) and potassium chloride (KCl) with a purity of 99% and potassium dichromate ($K_2Cr_2O_7$), with a purity of 100%.

Plants were transferred to glass containers containing 1.0 L of Hoagland's medium after the establishment of mass cultivation for acclimatization under bioassay condition at a temperature of 25 °C, lighting of 1.000 lux, and 12 hour photoperiod for 96 hours, as methodology adapted from OECD (2002).

Subsequently, the plants were transferred to 100.0 mL containers with 50.0 mL Hoagland's for 24 hours. A surface corresponding to an area of 19 mm^2 was sampled due to the small size of the test plant using a plastic duct with negative pressure made by a syringe and plunger. This operation was performed three times.

Reference substances were applied with 50 mL Hoagland's. Five acute toxicity tests (LC50;7d) were carried out for each reference substance, with concentrations of 0.03, 0.1, 0.35, 1.1, 3.6, 11.7, 38.03, and 123.58 g L^{-1} for NaCl, 0.15, 0.52, 1.97, 7.41, 27.9, and 104.25 g L^{-1} for KCl, and 1.0, 3.4, 11.6, 39.3, 133.6, and 454.3 mg L^{-1} for $K_2Cr_2O_7$, all of them with one control (without addition of the test substance) and three replications.

Acute toxicity tests for herbicides

The tested herbicides were diquat (Reglone®, 240.0 g L^{-1}), paraquat (Gramoxone, 200 g L^{-1}), imazapyr (Contain®, 266.3 g L^{-1}), 2,4-D (DMA®, 806.0 g L^{-1}), saflufenacil (Heat®, 700.0 g kg^{-1}), imazamox (Sweeper®, 700.0 g kg^{-1}), penoxsulam (Ricer®, 240.0 g L^{-1}), metsulfuron-methyl (Ally®, 700.0 g kg^{-1}), atrazine (Atrazina Nortox®, 500.0 g L^{-1}), and glyphosate (Rodeo®, 480.0 g L^{-1}).

Three tests of acute toxicity (LC50;7d) were performed for each herbicide, with diquat and paraquat concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, and 3.0 mg L^{-1}, glyphosate concentrations of 0.01, 0.1, 1.0, 3.5, 11.2, 36.5, and 118.0 mg L^{-1}, and concentrations for the other herbicides of 0.1, 1.0, 3.5, 11.2, 36.5, and 118.0 mg L^{-1}, all with one control and three replications under the same conditions for the test of reference substances.

Signs of toxicity were assessed at three, five, and seven days (OECD, 2002). Two methods of analysis were tested to assess *W. brasiliensis* response. The experimental unit was divided into four quadrants (25% of the surface area of the experimental unit), which were observed and had the percentage of chlorotic and necrotic plants estimated. This methodology was developed by Silva et al. (2012) for *A. caroliniana* and adapted for the assessment of *W. brasiliensis*. The other method consisted of counting healthy, chlorotic, and necrotic plants inside the container using a grid board placed on its top and taken to a stereomicroscope (LaboMed, Luxeo 4x). This method was developed in this study but was not feasible due to the small size of the test plant, which made it difficult to count during the assessment.

Mortality data were submitted to linear regression, and the lethal concentration (LC50;7d) was calculated by the software Trimmed Spearman-Karber (Hamilton et al., 1977). The ecotoxicological classification was performed according to USEPA (2017).

RESULTS AND DISCUSSION

Cultivation of *W. brasiliensis*

The assessment of mass cultivation showed that the best establishment of *W. brasiliensis* occurred using Oxisol + organic substrate of decaying aquatic plants in the greenhouse and laboratory. The mean plant life cycle was three months (establishment, growth, reproduction, and senescence). The standardization of methodologies of test plants cultivation is essential to harmonize the results and minimize their uncertainties (Brooks et al., 2015). Water quality variables, such as the pH and water hardness, and the presence of metals and organic substances in the cropping systems may be limiting factors in the development of sensitivity and toxicity responses of macrophytes and algae (Zwart and Posthuma, 2005).

The method established for cultivation was easy to handle and allowed the mass cultivation of the test plant, similar to methods that use culture media such as Hoagland's for *L. minor* (OECD, 2002) or *A. caroliniana* (Silva et al., 2012). Daughter leaves are produced by budding within a single pouch of the maternal matrix and several generations of leaves during the vegetative propagation, similar to that described for *W. microscopica* (Sree et al., 2015).

Sensitivity to reference substances

The five tests of sensitivity to sodium chloride (NaCl) showed that *W. brasiliensis* had a mean sensitivity of 15.61±2.13 g L^{-1}, with a mean 95% confidence interval between 12.05 and 19.46 g L^{-1} and a mean linear correlation between the tested concentration and toxicity (chlorosis/necrosis signs) of 78% (Table 1). The response of sensitivity to the reference substance NaCl of *W. brasiliensis* was lower than that of the standard plant *L. minor*, with a 50% growth inhibition (IC50;96h) of 6.87 g L^{-1} (Keppeler, 2009).

Table 1 - Sensitivity response (LC50;7d) of *W. brasiliensis* to sodium chloride (NaCl)

Test	Upper CI	LC50;7d	Lower CI	Linear equation	R^2
1	20.16	15.78	12.34	y = 11.7x − 22.2	0.78
2	18.47	14.40	11.22	y = 11.8x − 21.9	0.80
3	22.24	17.58	13.90	y = 11.5x − 23.2	0.75
4	21.71	17.15	13.55	y = 11.5x − 23.3	0.76
5	14.74	11.67	9.24	y = 11.9x − 22.6	0.81
Mean±SD	19.46±2.70	15.61±2.13	12.05±1.69	-	0.78±0.02

CI = 95% confidence interval; LC50;7d = 7 day lethal concentration 50%; SD = standard deviation.

Plants showed no signs of chlorosis and necrosis in the control. Concentrations of 0.03, 0.10, 0.35, 1.10, 3.60, 11.70, 38.03, and 123.58 g L^{-1} reached mortality means of 3.2, 5, 7.3, 17.2, 25.2, 33.8, 48.2, and 100%, respectively.

The sensitivity of *W. brasiliensis* to potassium chloride (KCl) was 21.81±0.49 g L^{-1}, with a 95% confidence interval between 13.29 and 17.02 g L^{-1} and mean linear correlation between the tested concentration and toxicity (chlorosis/necrosis signs) of 86% (Table 2). Potassium chloride had the best correlation between reference substances.

Plants showed no signs of chlorosis and necrosis in the control. Concentrations of 0.15, 0.52, 1.97, 7.41, 27.90, and 104.25 g L^{-1} led to chlorosis/necrosis of 1.3, 2.8, 9.9, 34.6, 49.6, and 91.6%, respectively.

Potassium dichromate ($K_2Cr_2O_7$) showed a medium sensitivity, with 7-d LC_{50} of 52.54±4.27 mg L^{-1}, a 95% confidence interval between 47.21 and 63.70 mg L^{-1}, and mean linear correlation between the tested concentration and toxicity (chlorosis/necrosis signs) of 81% (Table 3). Plants showed no signs of chlorosis and necrosis in the control. Concentrations of 1.00, 3.40, 11.6, 39.3, 133.6, and 454.3 mg L^{-1} reached mean mortality values of 6.5, 10, 12.1, 24.7, 86.0, and 91.6%.

Table 2 - Sensitivity response (LC50;7d) of *W. brasiliensis* to potassium chloride (KCl)

Test	Upper CI	LC50;7d	Lower CI	Linear equation	R^2
1	21.50	16.09	12.04	y = 17.2x − 28.1	0.93
2	22.74	16.70	12.26	y = 17.2x − 29.8	0.89
3	21.86	17.47	13.96	y = 17.4x − 28.6	0.85
4	21.58	17.38	13.99	y = 18x − 30.6	0.81
5	21.37	17.48	14.24	y = 18.1x − 32.6	0.82
Mean±SD	21.81±0.49	17.02±0.54	13.29±0.94	-	0.86±0.04

CI = 95% confidence interval; LC50;7d = 7 day lethal concentration 50%; SD = standard deviation.

Table 3 - Sensitivity response (mg L^{-1}) of *W. brasiliensis* to potassium dichromate ($K_2Cr_2O_7$)

Test	Upper CI	LC50;7d	Lower CI	Linear equation	R^2
1	59.46	48.58	39.69	y = 19.8x − 29.0	0.82
2	61.75	50.65	41.55	y = 20.71x − 34	0.79
3	70.97	60.28	51.20	y = 20.7x − 34.2	0.79
4	66.40	53.88	43.72	y = 19.8x − 29.4	0.83
5	59.93	49.32	40.59	y = 20.4x − 31.2	0.83
Mean±SD	63.70±4.38	52.54±4.27	47.21±7.46	-	0.81±0.01

CI = 95% confidence interval; LC50;7d = 7 day lethal concentration 50%; SD = standard deviation.

The species *W. brasiliensis* was less sensitive than *Spirodela polyrhiza* exposed to copper nanoparticles (EC50 = 1.15±0.09 mg L^{-1}), *L. minor* (EC50 = 0.84±0.12 mg L^{-1}), and *W. arrhiza* (EC50 = 0.64±0.05 mg L^{-1}) (Song et al., 2015), than *Ceramium tenuicorne* exposed to potassium dichromate with a EC50;48h of 3.9 mg L^{-1} (Macken et al., 2012), and similar response to *W. brasiliensis* exposed to zinc sulfate, with an EC50 = 21.64 mg L^{-1} (Lahive et al., 2011).

Thus, this plant presented an excellent sensitivity response to the three reference substances, indicating the potential for its use in biomonitoring programs of chemical substances such as herbicides (Hasenbein et al., 2017). In an analysis of databases on aquatic plant sensitivity, Lewis and Thursby (2018) found 76 species susceptible to copper compounds, 69 to atrazine, 48 to irgarol (antifouling algicide), and 46 to diuron. The species belonging to the family Lemnaceae were considered the most sensitive.

Acute toxicity for herbicides

Acute toxicity test for *W. brasiliensis* showed that the 50% lethal concentration (LC50;7d) of diquat was 0.07 mg L^{-1}, being classified as extremely toxic (USEPA, 2017). This result was similar to diquat for *L. minor* and *A. caroliniana*, with LC50;7d values of 0.01 and 0.02 mg L^{-1}, respectively (Garlich et al., 2016) and diquat for *L. minor*, with EC50;7d of 4.0 µg L^{-1} (Lewis and Trusby, 2018). Plants showed no signs of chlorosis and necrosis in the control and at concentrations of 0.01 and 0.05 mg L^{-1}, and 100% mortality at the other concentrations (0.1, 0.5, 1.0, and 3.5 mg L^{-1}).

The value of LC50;7d was lower than 0.05 mg L^{-1} for paraquat, being classified as extremely toxic (USEPA, 2017), similar to paraquat for *L. minor*, with an EC50;96h of 0.051 mg L^{-1} (Fairchild et al., 1997). Plants showed no signs of chlorosis and necrosis in the control. Mean mortality values of 55 and 95% were observed at concentrations of 0.01 and 0.05 mg L^{-1}, respectively. In addition, the other concentrations (0.1, 0.5, 1.0, and 3.5 mg L^{-1}) showed a 100% mortality.

The value of LC50;7d was 21.10 mg L^{-1} for imazapyr, with an upper limit of 26.68 mg L^{-1} and lower limit of 11.07 mg L^{-1}, being classified as slightly toxic (USEPA, 2017). This result is similar to that found for *A. caroliniana* (LC50;7d = 18.98 mg L^{-1}), differing from *Myriophyllum sibiricum* (IC50;14d = 0.032 mg L^{-1}) (Roshon et al., 1999) and *L. minor* (LC50;7d = 1.06 mg L^{-1}) (Cruz et al., 2015). Plants showed no toxicity signs in the control. Mean mortality values of 14% were observed at concentrations of 0.1, 1.0, and 3.5 mg L^{-1}, 22% at 11.2 mg L^{-1}, 65% at 36.5 mg L^{-1}, and 99% at 118.0 mg L^{-1}.

The 50% lethal concentration was not estimated for atrazine, 2,4-D, saflufenacil, imazamox, penoxsulam, and metsulfuron-methyl due to the absence of standardized mortality in relation to the increased concentrations. Thus, the LC50;7d was considered higher than 118.0 mg L^{-1} for these herbicides, which were classified as practically non-toxic (USEPA, 2017). The results presented by *W. brasiliensis* were similar to those of *L. trisulca* exposed to 3.0 mg L^{-1} of 2,4-D, showing no intoxication effect (Belgers et al., 2007) and *A. caroliniana* with a LC50;7d higher than 118.0 mg L^{-1} (Silva et al., 2012) LC50;7d = 100.0 mg L^{-1} of atrazine, bentazone + imazamox, and clomazone (Della Vechia et al., 2016).

For these herbicides, *W. brasiliensis* was less sensitive than *L. minor*, with EC50 of 0.80 mg L^{-1} of metsulfuron-methyl (Cedergreen et al., 2004), atrazine with LC50;7d of 5.27 mg L^{-1}, and bentazone + imazamox (31.58 mg L^{-1}) and clomazone (10.23 mg L^{-1}) for *L. minor* (Della Vechia et al., 2016), and atrazine with EC50 of 31.5 µg L^{-1} for *L. gibba* (Lewis and Trusby, 2018).

Plants showed no signs of chlorosis and necrosis for 2,4-D in the control and at a concentration of 0.1 mg L^{-1}. Also, plants showed a similar percentage of mortality (8.7%) at 1.07, 3.5, 11.16, 36.4, and 118.0 mg L^{-1}. Imazamox in plant control did not promote chlorosis/necrosis signs. Mortality values of 7.5% were observed at 0.1, 1.0, 3.5, and 11.2 mg L^{-1}, 14% at 36.5 mg L^{-1}, and 40% at 118.0 mg L^{-1}. Penoxsulam showed no chlorosis/necrosis signs since all tested concentrations had 7.75% mortality. Atrazine also promoted no chlorosis/necrosis, but mortality values of 11% were observed at 0.1, 1.0, and 3.5 mg L^{-1} and 16.1% at 11.2, 36.5, and 118.0 mg L^{-1}.

The sensitivity of *W. brasiliensis* was lower for glyphosate (Rodeo®) (LC50;7d > 118.0 mg L^{-1}) when compared to glyphosate (Roundup®), with a LC50;7d of 46.9 mg L^{-1} for *L. minor* (Cedergreen and Streibig, 2005); for glyphosate (Roundup®), it was 11.20 mg L^{-1}; for *L. gibba* (Sobrero et al., 2007) and glyphosate (Scout®), it was 23.66 mg L^{-1}, and for glyphosate (Trop®), the LC50;7d was 38.91 mg L^{-1} for *A. caroliniana* (Silva et al., 2012).

Plants showed no chlorosis/necrosis signs after exposure to glyphosate (Rodeo®). The concentrations 0.01, 0.1, 1.07, 3.4, 11.6, and 36.4 mg L^{-1} had mortalities similar to 26.3% and 118.0 mg L^{-1} to 50%.

Toxicity response of *W. brasiliensis* to herbicides indicates that this plant presents a response relatively similar to that of other plants standardized or used in toxicity or environmental biomonitoring studies. *W. brasiliensis* can be considered extremely sensitive to diquat and paraquat, sensitive to imazapyr, and little sensitive to the other herbicides. The response capacity for the different groups of herbicides, the standardized response to reference substances, and ease of cultivation in the laboratory indicate the potential for using this aquatic plant in the assessment of herbicide risk in aquatic environments.

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