



AGRARIAN SCIENCES

Morphoanatomical injuries in *Pistia stratiotes* L. (Araceae) as a result of exposure to clomazone in water

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Abstract: Contamination of water sources due to herbicide is of great concern. Clomazone is a pesticide with a high contamination potential which could possibility lixiviate to water streams. Changes caused by residual herbicide include flora modifications which are generally detrimental for some species. The lack of morphological studies performed in aquatic plants exposed to herbicide-contaminated environments has encouraged the development of our research. For the first time, we present a study that aimed to evaluate leaf injuries visible to the naked eye as well as microscopical effects which may be caused by clomazone on *Pistia stratiotes*. *Pistia stratiotes* was subjected to five concentrations of clomazone. Our analysis showed leaf injuries, especially after 15 days of clomazone application. Hormesis was observed when the water lettuce was subjected to the lower concentrations. Total leaf area showed increase following by reduction while injured until reaching the highest concentration. Although the concentrations of clomazone tested in our study are not lethal to water lettuce, such herbicide have still caused morphoanatomical damages on leaves which advocates for the use of *P. stratiotes* as a bioindicator of the presence of herbicides such as clomazone in water.

Key words: aquatic macrophyte, clomazone, herbicide, hormesis, leaf anatomy, water lettuce.

INTRODUCTION

A pesticide is any substance or mixture of substances of chemical or biological ingredients intended for repelling, destroying or controlling any pest, or even regulating plant growth (FAO/WHO 2016). Worldwide pesticide production increased at a rate of more than 10% per year. From 1950 to 2000, the amount of pesticides used worldwide jumped from 0.2 million tons to more than 5 million tons (Carvalho 2017). In 2014, the Brazilian market of pesticides had grown 190% if compared to 93% growth of the global market, setting Brazil as a leader in the worldwide ranking of pesticide usages since

2008 (Rigotto et al. 2014). According to Agência Nacional de Vigilância Sanitária (ANVISA 2013), in the 2010/2011 harvest, there was a consumption of 936,000 tons of pesticides in Brazil, which earned US\$ 8.5 billion to enterprises that control 75% of that market (Rigotto et al. 2014). The release of genetically modified seeds and its spreading in agricultural areas are related to the subsequent increases in pesticides consumption, being the use of herbicides responsible for 45% of consumed volume, followed by fungicide (14%) and insecticide (12%) (Rigotto et al. 2014).

With the intensification of production in areas where water basins are found, comes

great concern, resulting from herbicide use (Grützmacher et al. 2008). The aquatic environment is the main drain of pesticides (Britto et al. 2012). Contamination of both surface and ground water by pesticides is reported all over the world (Turgut 2003, Zhou et al. 2006, Lari et al. 2014) and it is one of the greatest environmental concerns (Konstantinou et al. 2006). When pesticides reach freshwater ecosystems, they may interact with both biotic and abiotic components, although abiotic factors can lead to its degradation, the interaction with the biotic parts may lead to hostile effects on the freshwater community (Schäfer et al. 2011). Although, pesticide occurrences in agricultural catchments will rarely lead to acute extermination of the majority of species (Cedergreen & Rasmussen 2017), one cannot deny that peak pesticide concentrations can affect the species communities on sub-lethal endpoints which ultimately may lead into altered community structure and function (Schäfer et al. 2011, Cedergreen & Rasmussen 2017).

Regarding herbicide molecules with contaminant potential of water streams, clomazone {2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone} is known as a potential threat to aquatic ecosystem (Van Scoy & Tjeerdema 2014). This herbicide has high water solubility (1.1 mg L⁻¹ at 23 °C) (Van Scoy & Tjeerdema 2014) and is categorized as a non-persistent pesticide, which DT50 value is 22.6 days (PPDB 2019). However, clomazone persistence in the soil may be over 150 days (EPA 2007). When applied to the soil it may lixiviate and reach deep layers in the soil (Rodrigues & Almeida 2011) as well as surface and underground water (Caldas et al. 2010, Santos et al. 2015). Santos et al. (2015) detected clomazone in all collecting seasons in over 60% of water samples (i.e. a total of 32 wells and 13 water springs sampled). Such herbicide was detected at depths up to

160 m, which brings concerns for the potential impacts of clomazone on drinking water systems. Degradation and mineralization of clomazone seems to be biologically dependent (Mervosh et al. 1995) with major microbial transformation reactions involving hydroxylation (Liu et al. 1996). Moreover, monitoring of the clomazone in water samples has revealed that clomazone persists in agricultural water for at least 130 days, being present in 90% of the river water samples analyzed (Zanella et al. 2002). Several studies have investigated and found the presence of clomazone in waters (Caldas et al. 2011).

Clomazone is an isoxazolidinone acting directly in the plant photosynthetic process, impairing the formation of photosynthetic pigments in the biosynthetic pathway of carotenoids (Ferhatoglu & Barrett 2006, Van Scoy & Tjeerdema 2014), which play an important role protecting the plant's photosynthetic apparatus against harmful effects of light intensity (Merzlyak et al. 1999, Schreiber et al. 2013). Effects in human erythrocytes and the possible formation of reactive oxygen species have also been demonstrated (Santi et al. 2011).

Considering the amounts of herbicides annually applied for weed management and the necessity to constantly monitor environmental contamination, it is important to evaluate the quality of water as residual herbicides may reach water streams. Biomonitoring using aquatic macrophytes comes with several advantages when compared to other methods for monitoring water quality (Pedralli 2003). Macrophytes can be used as bioindicators to provide a secure and reliable evaluation of the environmental quality. Macrophytes are good bioindicators of changes occurring in water bodies as a result of human-induced interferences (Lukács et al. 2009), on account of its practical application and low cost, hence being an efficient alternative to chemical

and physical water analyses (Pita-Barbosa et al. 2009).

Leaf injuries visible to the naked eye stand out as the most used method to evaluate plant species sensitivity to several stress factors. This evaluation frequently requires validation through microscopy analysis (Reig-Armiñana et al. 2004, Pita-Barbosa et al. 2009). In the past years, researchers have used plant anatomy as a tool to study the effects of pollutants in plants (Sant'Anna-Santos et al. 2006, Sant'Anna-Santos & Azevedo 2007, Pedroso & Alves 2008). Microscopic evaluations are essential for an early diagnosis of damage even before such damages are visible on the outer plant body by the naked eye, especially contributing to elucidate phytotoxicity mechanisms (Sant'Anna-Santos & Azevedo 2007).

Because of the water solubility of clomazone, the potential impact of such a herbicide on surface water, groundwater and aquatic organisms is of great concern. The lack of morphoanatomical studies performed in aquatic plants exposed to herbicide-contaminated environments has encouraged the development of our research. As far as we are concerned, there are no reports on the literature regarding the effect of clomazone on *Pistia stratiotes* L. (Araceae), popularly known as water lettuce. For the first time, we present a study that aimed to evaluate leaf injuries visible to the naked eye as well as the microscopical effects which may be caused by clomazone on water lettuce. Our hypothesis is that even small concentrations of clomazone (i.e. 0.037, 0.111, 0.333 and 1.0 mg L⁻¹) may still be able to cause leaf injuries on macrophytes.

MATERIALS AND METHODS

Pistia stratiotes specimens were collected in a lake in the Municipality of Diamantina

(Fig. 1) (State of Minas Gerais, Brazil). There is no historic of contamination issues in this area. Plants collected were kept in 5 L plastic pots with tap water at pH 6.5. The new clones produced at the ends of stolons were collected when rosettes presented 15 cm in diameter, in order to make the experimental design more consistent. The clones were then transferred to plastic pots (two plants per pot) under hydroponic systems with nutrient solution as described by Castellane & Araújo (1995). The pH of the solution was daily checked with a portable pH meter (model PHTEK) and corrected to 6.5 when necessary. Nutrient solution was not renewed during acclimatization. Plants were acclimatized for 15 days and then subjected to five treatments with the following clomazone concentrations diluted in water (Gamit 360g a.i. L⁻¹) (Gamit 360 CS: Batch 24-17: Manufactured, Dec 2019; Expiration, Dec 2019): 0.0; 0.037; 0.111; 0.333; and 1.0 mg L⁻¹.

Automatic pipettes with a volumetric capacity of 1 mL were used for the application of clomazone in the plastic pots holding 5 L of water. Standard solutions of clomazone were prepared in stock solution in 10 mmol L⁻¹ CaCl₂ as diluents. Clomazone was applied 2 h after transferring the plants to pots. In this way, it was possible to obtain concentrations at 0.037, 0.111, 0.333 and 1.0 mg L⁻¹ in the water within pots. After clomazone application, a composite sampling of the pots was performed to ensure that initial concentrations corresponded to each treatment proposed.

The experiment was carried out in a greenhouse at Universidade Federal dos Vales do Jequitinhonha e Mucuri – Diamantina (Minas Gerais, Brazil) from March to May 2015 under controlled temperature and humidity conditions set at ±24 °C and 78%, respectively, through a system of continuous aeration. A completely

randomized block design with four replicates per concentration was used.

Injuries were daily observed and documented by means of photos taken with a mobile phone (Samsung Galaxy A3) equipped with a 13 Mp digital camera of 4128 x 3096 pixel resolution.

At day 7 and 15 after the herbicide application, samples from the leaf middle area of fully expanded leaf areas that were apparently healthy (i.e. green areas) were collected and fixed in FAA50 (formaldehyde, acetic acid and 50% ethanol; 1:1:18 by volume) (Johansen 1940), being later stored in 70% ethanol. Two ~0.5 cm² fragments were taken from the samples of each replicate, dehydrated through a graded ethanol series from 70 to 95% and embedded in methyl methacrylate resin (Historesin®, Leica Instruments, Heidelberg, Germany) according to the manufacturer. Cross sections at 5 µm thickness were made in a manual rotary microtome Model 80 (American Optical scientific instruments) - USA using stainless steel blade. Sections were stained with toluidine blue at pH 4.4 (O'Brien & McCully 1981), dried at room temperature and mounted in synthetic resin dissolved in toluene (Permount, Fisher Scientific, NJ, USA). At least three slides with 15 sections each were made for each replicate. Observation and photographic record were carried out in a Primo Star, Zeiss® light microscope equipped with a digital camera (AxioCam ERc5s). Three photos for each slide were taken.

The general morphological characterization of the leaf structure of the species studied was performed using leaves not subjected to clomazone application. For the micromorphometrical analyses, linear measurements of the following characters were accounted by using ANATI QUANTI software, version 2.0 for Windows® (Aguiar et al. 2007):

thickness of leaf blade, palisade parenchyma, adaxial epidermis, and abaxial epidermis.

Moreover, 21 and 45 days after application, the level of injury was determined in percentage of visible injuries occupying the rosette area. Evaluation of fresh mass increment was performed weighing the whole plants at 0, 7, 14, 21 and 28 days after application of the herbicide in a precision scale (SHIMADZU, BL3200H). The means obtained were transformed into percentage and fresh mass increment was calculated in relation to the difference of the fresh mass taken in the first weighing (at day 0) which was set at 100%.

At 45 days, all leaves were collected and digitalized in SAMSUNG printer (LASERJET SCX-4200). Determination of total leaf area (TLA) and injured leaf area (ILA) were also obtained by using the program ANATI QUANTI, version 2.0 for Windows® (Aguiar et al. 2007).

After the last plant collection, samples of the nutrient solution and of the plant material were analyzed for detection and quantification of clomazone residues as described by (Queiroz et al. 2012). We made use of the based on liquid chromatography triple quadrupole tandem mass spectrometry (LC-MS/MS): HPLC Finnigan Surveyor Thermo Scientific TSQ Triple Quadrupole LC-MS Systems. The volume of injection was 10 µL and the mobile phase was acetonitrile and 1% acetic acid in gradient mode. Recovery tests (n = 3) were performed using a fortification level of 0.5; 1.0 and 2.0 mg Kg⁻¹ adding a standard solution of 5.0 mg mL⁻¹ in methanol (Queiroz et al. 2012). The results were subjected to variance analysis and interpreted using regression test at 5% level of significance.

RESULTS

Clomazone residues were not found in solutions at lower concentrations of clomazone (0.037; 0.111 mg L⁻¹) and in samples of plant tissue. On the other hand, for concentrations at 0.333 and 1.0 mg L⁻¹, it was found 0.019 and 0.048 mg L⁻¹ of clomazone residues in the nutrient solution, respectively. However, clomazone residues were not found in plant material subjected to such concentrations of clomazone.

When exposed with clomazone, water lettuce showed bleaching of young leaves which then evolved to necrosis, except for the lowest concentration tested, 0,037 mg L⁻¹ (Fig. 2). Plants subjected to the lowest concentration of clomazone tested, 0.037 mg L⁻¹, did not show any symptoms in the rosette (Fig. 2b). However, injuries occupying 25% of the rosette area were observed when subjected to concentrations at

0.111 mg L⁻¹ (Fig. 2c) while injuries occupying 50 to 75% of the rosette area were observed in plants exposed to 0.333 and 1.0 mg L⁻¹, respectively (Fig. 2d, e).

Total leaf area and injured leaf area analyses indicated lower mean value of total leaf area (17 cm²) for plants growing at 0.52 mg L⁻¹ concentrations of clomazone and higher mean value of injured leaf (10.21 cm²) at 0.68 mg L⁻¹ concentrations (Fig. 3). Water lettuce showed increase of fresh mass following the days of evaluation, being represented in the graph by straight lines. The highest values were observed for plants growing at 0.037 mg L⁻¹ concentration which presented an equation with the highest slope coefficient (2.34) (Fig. 4).

Anatomically, the leaf blades of water lettuce are constituted by a single-layered epidermis with tabular to rounded cells and pluricellular-uniseriate trichomes on both leaf

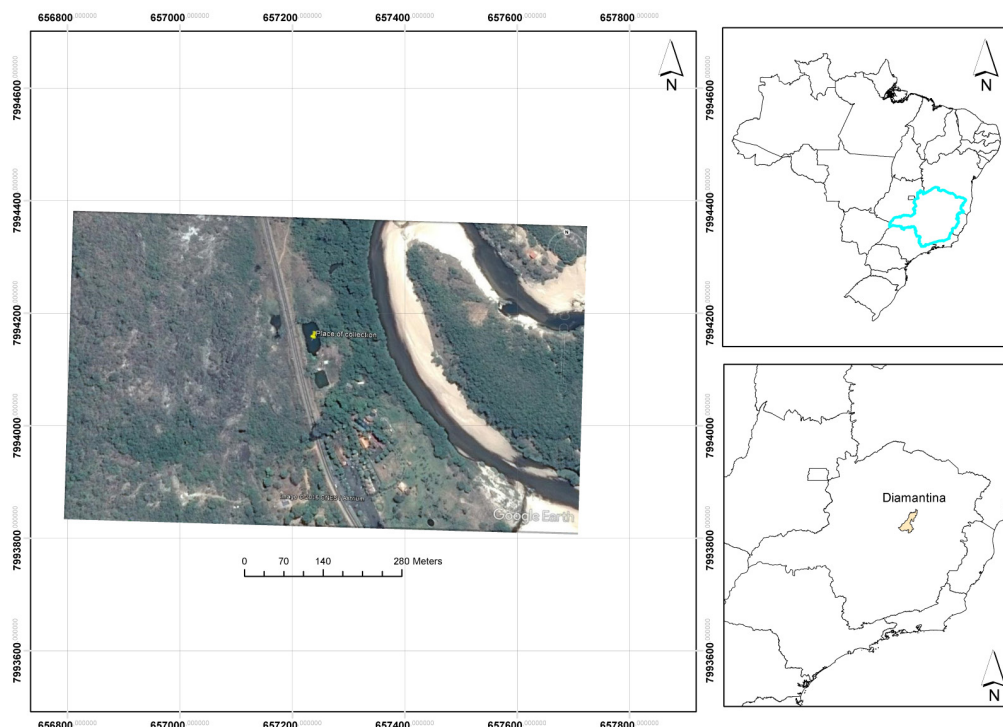


Figure 1. Area of collection of *P. stratiotes* at Diamantina, Minas Gerais State, Brazil.

surfaces (Fig. 5a). Leaves are amphistomatic and mesophyll is dorsiventral (Fig. 5a), made up by a palisade parenchyma turned to adaxial side (Fig. 5a) and aerenchyma to the abaxial side, as the air-gaps are involved by

braciform cells that interrupt the intercellular spaces. Idioblasts with calcium oxalate crystals (raphides and druses) are observed in the palisade parenchyma and aerenchyma (Fig. 5a). Collateral vascular bundles are placed in the

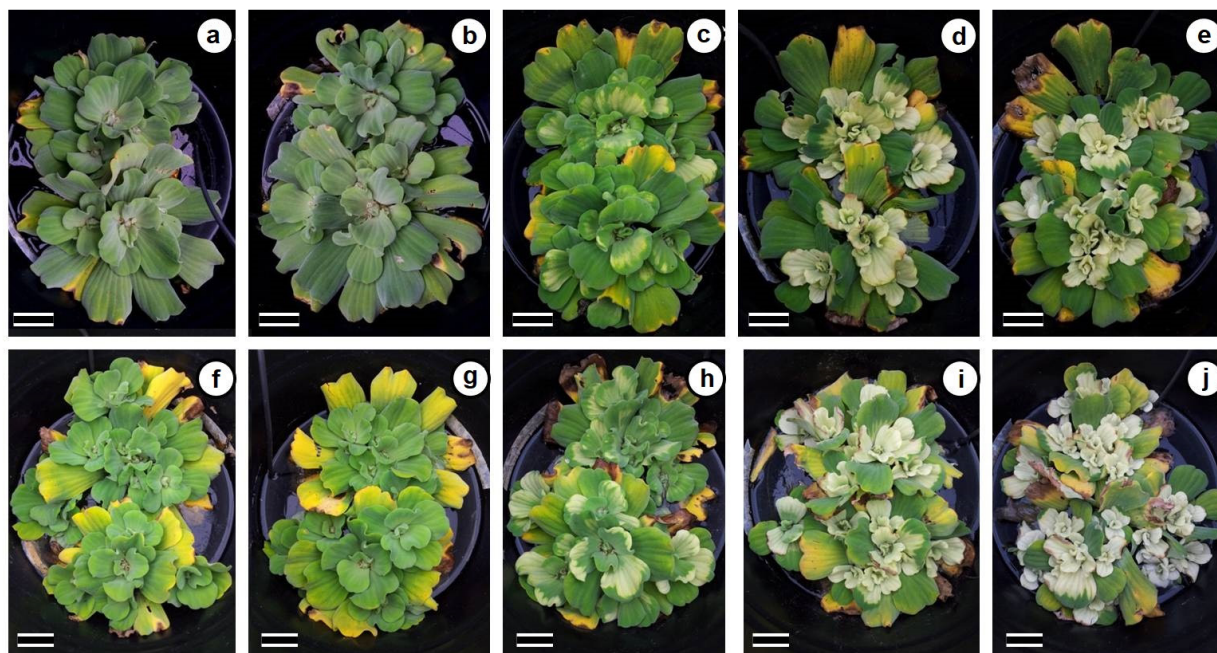


Figure 2. *Pistia stratiotes* injuries observed at 21 (a-e) and 45 (f-j) days after clomazone application. a and f. Control 0.0 mg L⁻¹; b and g. 0.037 mg L⁻¹; c and h. 0.111 mg L⁻¹; d and i. 0.333 and e and j 1.0 mg L⁻¹. Scale bar = 5 cm.

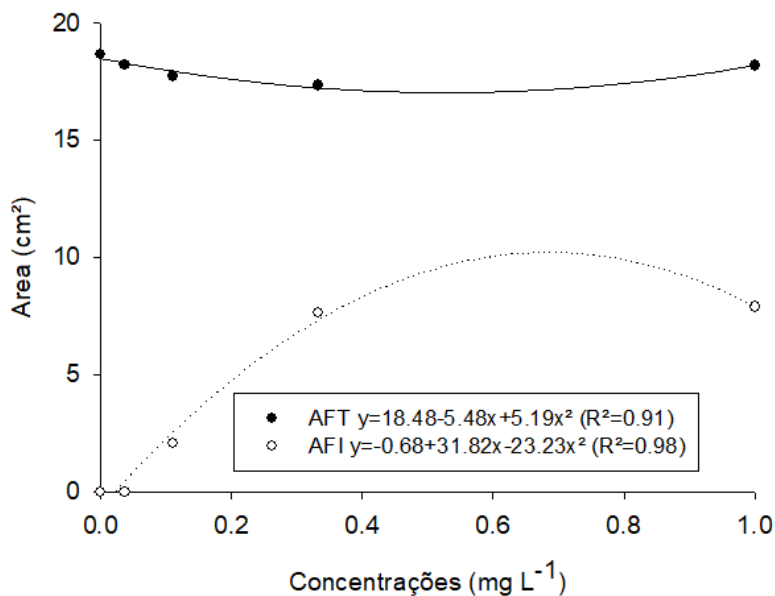


Figure 3. Total leaf area (TLA) and injured leaf area (ILA) of *P. stratiotes* at 45 days after clomazone application.

median area of the mesophyll enclosed in a double bundle sheath (Fig. 5a).

Regarding the micromorphometrical analyses, the variable leaf blade thickness did not show statistical significance at 7 days, with mean value of 299.32 μm , as it may be observed in anatomical sections (Fig. 5b, c). However, at 15 days, it was observed an increase in the leaf blade thickness up to 0.49 mg L^{-1} of clomazone, following a reduction of thickness up to 1.0 mg L^{-1} (Fig. 5d, e and Fig. 6a). Palisade parenchyma thickness showed concave downward response curves at 7 and 15 days with the highest values (116.26 and 108.0 μm) observed at the concentrations of 0.64 and 0.57 mg L^{-1} , respectively (Fig. 6b).

Changes on the adaxial epidermis thickness of leaves collected at 7 days were not statistically significant with mean of 19.78 μm as observed in anatomical sections of plants subjected to 0.111 and 1.0 mg L^{-1} (Fig. 5b, c) which made it unsuitable to an adequate model. On the other hand, for leaves collected at 15 days, higher values for

adaxial epidermis thickness (21.87 μm) were accounted at the 0.54 mg L^{-1} concentration of clomazone (Fig. 6c), as observed in anatomical sections of plants subjected to 0.037 and 1.0 mg L^{-1} (Fig. 5c, d).

Abaxial epidermis thickness of leaves collected at 7 days was not statistically significant with a mean value of 29.34 μm (Fig. 5b, c). However, leaves of plants subjected to at 0.67 mg L^{-1} concentration of clomazone and collected at 15 days showed lower mean values for thickness (20.5 μm) (Fig. 6d), as observed in anatomical sections of plants subjected to 0.037 and 1.0 mg L^{-1} (Fig. 5c, d).

DISCUSSION

As clomazone residues were not found in plant tissues of individuals subjected to any of the solutions tested, one plausible explanation for this is that the residual clomazone which would be present in the plant tissues was

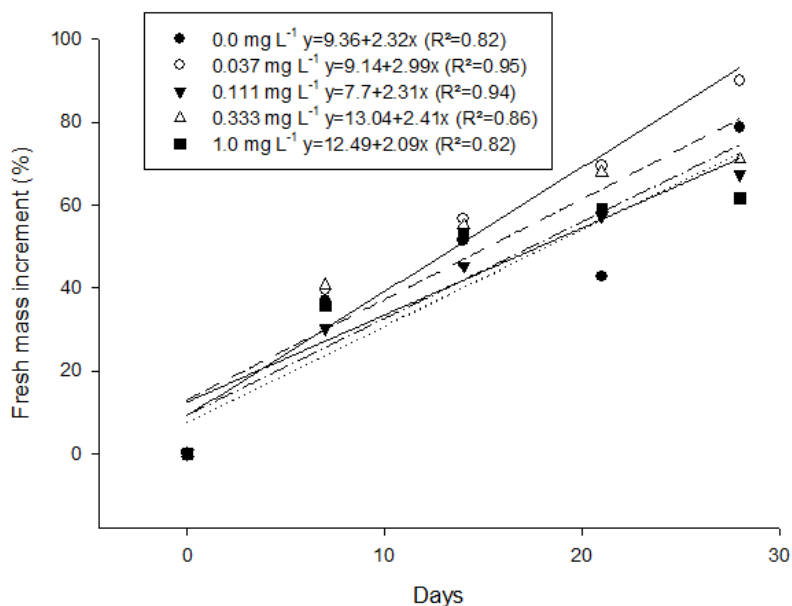


Figure 4. Fresh mass (%) of *P. stratiotes* following clomazone application. Means of values of fresh mass refer to control (without herbicide): 0 day = 123.75 g; 7th day = 169.46 g; 14th day = 187.52 g; 21st day = 176.53 g and 28th = 221.03 g.

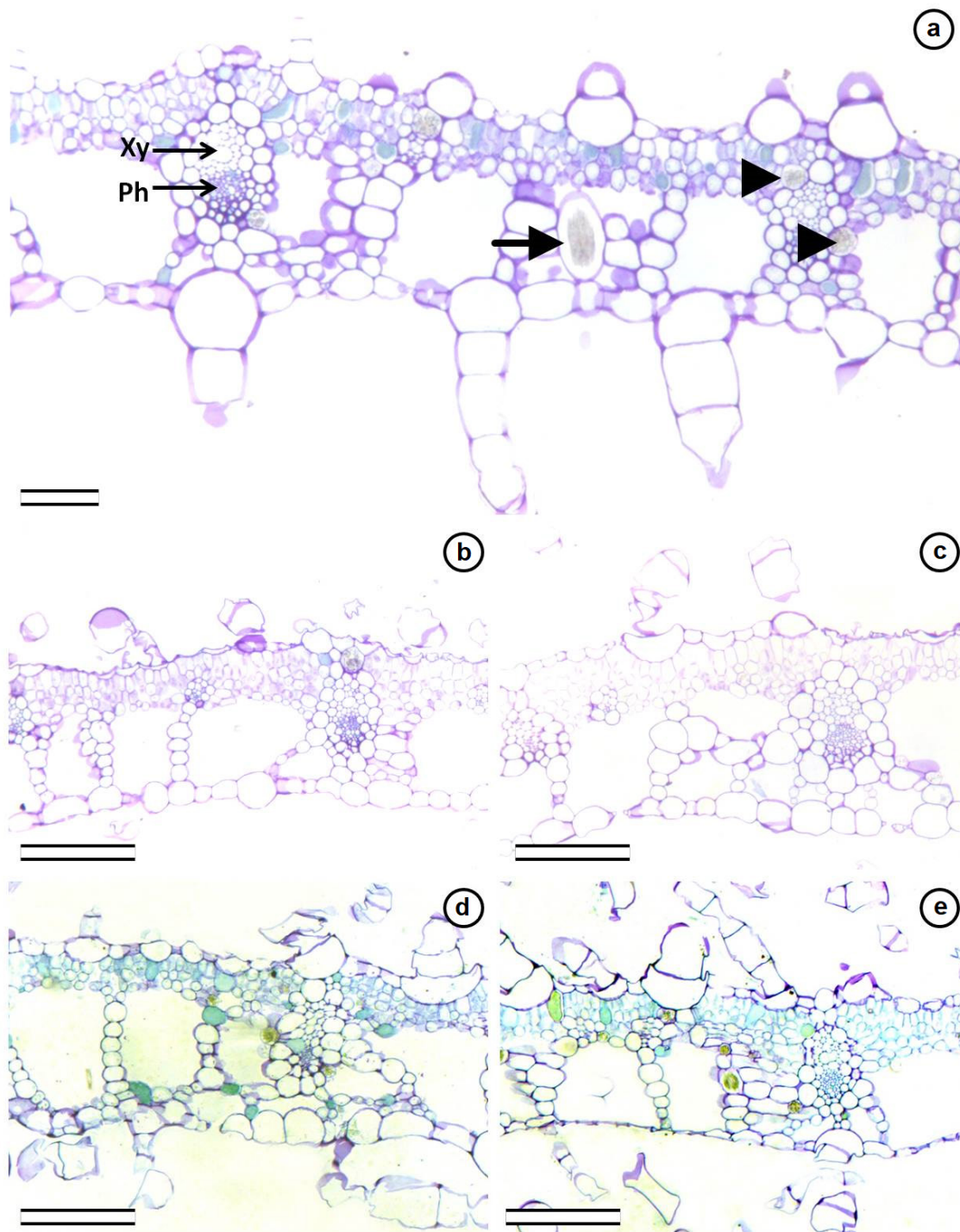


Figure 5. Cross sections of leaf blade of *P. stratiotes* subjected to clomazone application. a. Control; b and c. Plants subjected to 0.111 and 1.0 mg L⁻¹ at 7 days after clomazone application. Note that the thickness of leaf blade does not change significantly regardless of the concentration of clomazone applied. d and e. Plants subjected to 0.037 and 1.0 mg L⁻¹ at 15 days after clomazone application. Note that the abaxial epidermis in e is much thinner than in d while the palisade parenchyma in e is thicker than in d. arrowhead = druse; arrow = raphide; Ph = phloem; Xy = xylem.

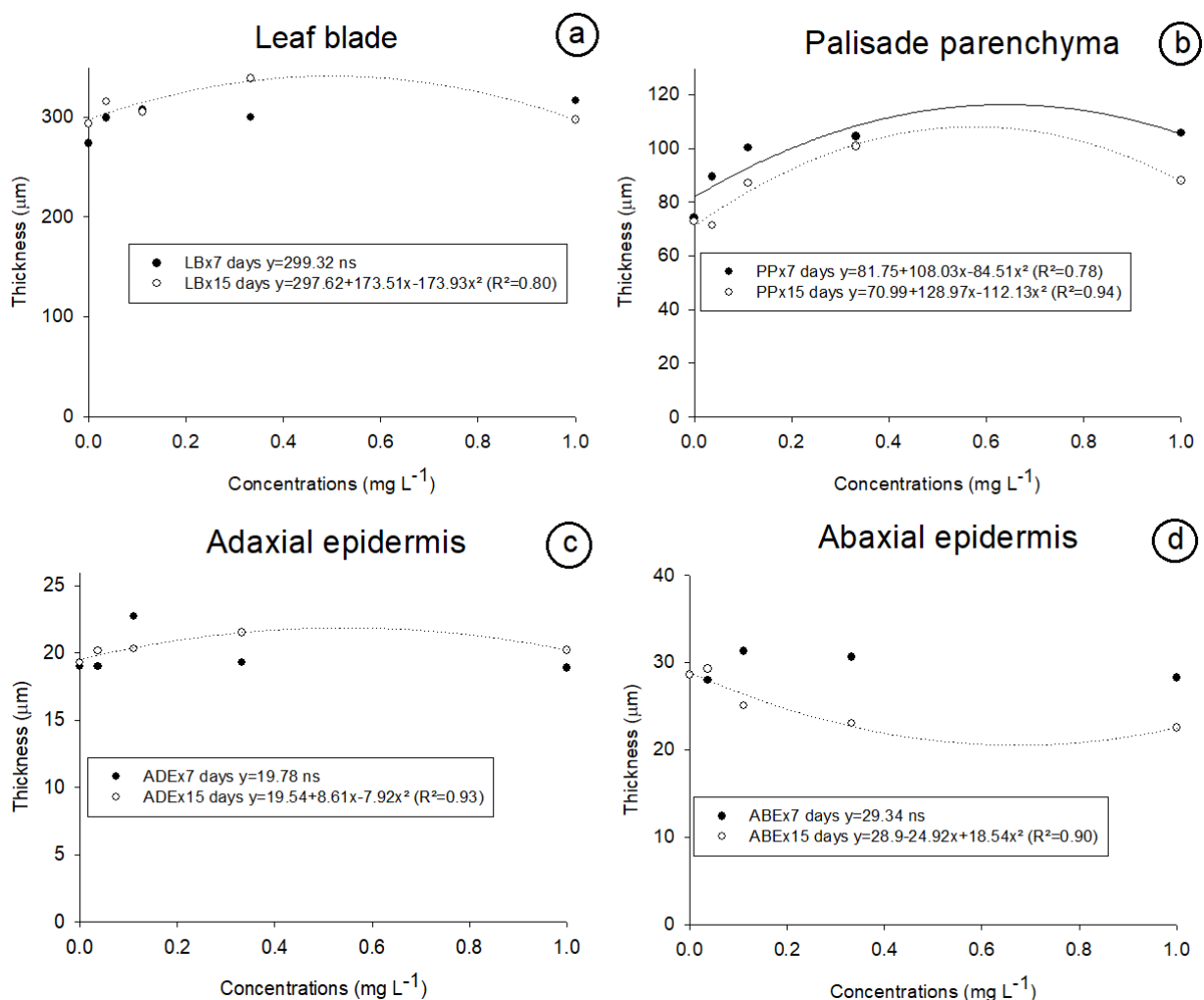


Figure 6. Thickness of leaf tissues of *P. stratiotes* obtained from cross sections of leaf blades collected at 7 and 15 days after clomazone application. **a.** Leaf blade thickness; **b.** Palisade parenchyma thickness; **c.** Adaxial epidermis thickness; **d.** Abaxial epidermis thickness.

actually below the limit of detection. There is also the possibility of forming metabolites derived from clomazone and such metabolites were not screened in our study. Liu et al. (1996) have proposed a microbial breakdown route for clomazone. Metabolites such as 5-hydroxyclozomazone, hydroxymethylclomazone, 2-chlorobenzyl alcohol and 3'-hydroxyclozomazone were identified as metabolites derived from the clomazone breakdown by common soil fungus and bacterium. Clomazone photodecomposition may also take place, with formation of

four major photoproducts which were tentatively identified as 2-chlorobenzamide, N-hydroxy-(2-benzyl)-2-methylpropan-amide, 2-[2-phenol]-4,4-dimethyl-3-isoxazolidinone and 2-[(4,6-dihydroxyl-2-chlorine phenol)]-4,4-dimethyl-3-isoxazolidinone (Cao et al. 2013). Degradation of clomazone under UV light is faster (half-life of 51–59 min) when compared to sunlight (half-life of 87–136 d) (Cao et al. 2013). Metabolites such as hydroxyclozomazone, hydroxymethylclomazone and 5-keto-clomazone

were also reported to be found in plants (PPDB 2019).

Water lettuce showed bleaching of young leaves which then evolved to necrosis, except for the lowest tested concentration. Bleaching is a characteristic symptom of plants sensitive to this group of herbicide (i.e. isoxazolane) (Senseman 2007). Clomazone mode of action promotes lipid peroxidation in cells due blocking of carotenoid synthesis in emerging shoot, resulting in a characteristic bleaching of new leaf tissues (Yasuor et al. 2008). The macrophyte *Azolla caroliniana* Willd. also presented injuries when exposed to clomazone concentrations ranging from 0.6 to 0.8 mg L⁻¹ (Silva et al. 2012). Therefore, the sensitivity of water lettuce when exposed to clomazone is evident as even low concentrations of this product results in plant intoxication.

Leaf area is of great importance for modelling growth and development of plants (Lima et al. 2012). Total leaf area and injured leaf area analyses indicated lower mean value of total leaf area for plants growing at 0.52 mg L⁻¹ concentrations of clomazone while higher mean value was found at 0.68 mg L⁻¹. Leaf surface expansion provides a better use of sunlight and therefore an increase in the photosynthetic capacity which makes it a good feature to evaluate plant growth (González-Sanpedro et al. 2008).

However, this study showed that water lettuce increases in injured leaf area because of clomazone action. The mechanism of action of this herbicide acts in the pathway of the carotenoid biosynthesis. The carotenoids are responsible for protecting leaves against excessive light intensity as light intercepted by leaves without such photo-protectors may cause photo-oxidation and chlorophyll eradication (Takahashi et al. 2009). Such a fact highlights

the herbicide action which indirectly interferes on photosynthesis leading to bleaching.

Although water lettuce showed increases of fresh mass after being subjected to clomazone, such increase cannot be actually related to a good quality gain in fresh mass as bleaching clearly worsens as the concentration become higher and the days pass by. The possible evidence for the cause of the higher allocation of fresh mass under low concentration effect is the hormesis effect. Calabrese & Blain (2009) argues that hormesis is a dose response phenomenon causing stimulatory effects at lower doses while at higher doses inhibitory. Such relation has quantitative features that are precise, as there are well established boundaries between stimulating and toxic effects.

Considering that the action mechanisms of clomazone acts in the elimination of the precursor of carotenoids, low herbicide concentrations (as applied in our study) may actually stimulate plants to produce new compounds without promoting excessive damage to the area that has already been synthesized. In such a way, the plant could become more tolerant to photo-damages and consequently have chlorophyll better protected. The final effect would be a better allocation of resources, based on light use, supported by increases in fresh mass. Velini et al. (2008) reported growth stimulation on vegetative organs of soybean, eucalyptus, corn, pinus, coffee and citrus, when low doses of glyphosate simulating herbicide drift were applied.

The variable leaf blade thickness did not show statistical significance at 7 days. However, at 15 days after being subjected to clomazone, an increase in the leaf blade thickness was observed, following a reduction of thickness. Palisade parenchyma thickness showed concave downward response curves. Leaf blade thickness is generally determined by the

expansion of photosynthetic tissue (Rôças et al. 1997), for example, the increase of the palisade parenchyma layers (Rôças et al. 1997, Cao 2000). As the photosynthetic tissues increases when exposed to clomazone concentrations up to 0.49 mg L^{-1} , it seems that the areas in the leaf that are still apparently healthy (i.e. green when observed with the naked eye) try to compensate for other leaf areas that are not photosynthetic active (i.e. areas that are suffering with bleaching). Besides, light can be propagated through the central vacuole of the palisade cells and through air spaces between the cells, distributing light more uniformly to chloroplasts within the leaf (Vogelmann & Martin 1993). However, in concentrations of clomazone around 1.0 mg L^{-1} , the photosynthetic tissue decreases, supporting the idea that the plant is not able to compensate the issues caused by the bleaching. Palisade parenchyma thickness is also important to protect leaves against high luminous radiation (Tuffi Santos et al. 2008).

Palisade parenchyma thickness plays an important function to protect leaves against high luminous radiation (Tuffi Santos et al. 2008), as well as leaf blade thickness. Therefore, water lettuce probably increases the palisade parenchyma and leaf blade thickness as a mechanism of defense against the light intensity, since the plants are growing in solution treated with clomazone which destroy carotenoid biosynthesis and then reducing the ability of these pigments to dissipate the excess of energy from light in the plant shoot (Odero et al. 2015).

Changes on the adaxial epidermis thickness of leaves collected at 7 days were not statistically significant in plants subjected to 0.111 and 1.0 mg L^{-1} which made it unsuitable to an adequate model. On the other hand, for leaves collected at 15 days, higher values for adaxial epidermis thickness were accounted at the 0.54 mg L^{-1} concentration of clomazone. As

discussed by Cen & Bornman (1993), an increase in the adaxial epidermis thickness determines the leaf precautionary effect towards water loss and mitigation of sun rays penetration in the leaf blade. Studies demonstrate that the main barrier to herbicide penetration in leaves is the occurrence of thicker adaxial epidermis and cuticle (Procópio et al. 2003). Adaxial epidermis plays important functions such as leaf surface protection against excessive light, control of gas exchanges and water vapor, as well as protection of the mesophyll (Dickson 2000, Evert 2006). Therefore, our results can be explained in the light of defense mechanisms as the studied species attempts to amend and overcome injurious effects caused by luminous intensity along with herbicide action. Our study indicates that water lettuce possibly has increased the adaxial epidermis thickness as a defense mechanism attempting to reduce the damage caused by the light with herbicide action. Thus, this thicker tissue assists to protect the whole mesophyll.

Although abaxial epidermis thickness of leaves collected at 7 days was not statistically significant, leaves of plants growing at 0.67 mg L^{-1} concentration of clomazone which were collected at 15 days showed lower mean values for thickness. Due to its physical-chemical characteristics, clomazone is relatively volatile with highly vapor pressure (19.2 mPa at $25 \text{ }^\circ\text{C}$) (Senseman 2007, Rodrigues & Almeida 2011, Van Scoy & Tjeerdema 2014, PPDB 2019). It is possible that the clomazone that would be lost by volatilization was actually kept under the abaxial side of the leaf surface for a while in a way that the abaxial side of leaf surface acted as a barrier to the escape of clomazone to the atmosphere. That may be the reason why a reduction on the thickness of the abaxial epidermis was observed.

Even with absorption of the herbicide by target plants, residues of the clomazone may leach and reach groundwater yielding a potentially hazardous impact. The lowest concentration of clomazone tested was 0.037 mg L⁻¹ which would be equivalent to 26 g ha⁻¹. Considering that the label rate for clomazone application is 360 g a.i. ha⁻¹ (up to 1260 g a.i. ha⁻¹) (MAPA 2017), even 0.037 mg L⁻¹ which is equivalent to ~3% of the average recommended dose by the manufacturer would still injure plants.

Water lettuce was more sensitive when exposed to 0.333 and 1.0 mg L⁻¹ clomazone concentrations, presenting higher degrees of intoxication. Anatomical studies showed that 15 days after plants exposure to the herbicide would be the adequate timing to perform micromorphometric evaluations as the evidences of tissue modifications are better observed at that time. Although our anatomical study was only based on leaves, the necessity to evaluate how roots are affected by herbicides is at demand as such organ may also be affected by the uptake of herbicides, as shown for *Pinus radiata* D. Don. (Marks & Becker 1990).

CONCLUSION

Although the concentrations of clomazone tested in our study are not lethal to water lettuce, such herbicide have still caused morphoanatomical damages on leaves which advocates for the use of *P. stratiotes* as a bioindicator of the presence of herbicides such as clomazone in water. However, future studies evaluating the sensitivity of other organs, such as roots, to clomazone could indicate which organ is more suitable for biomonitoring of waters when using *P. stratiotes*. Studies subjecting *P. stratiotes* to other herbicides would confirm whether such species is affected by other herbicides or only clomazone. Although the acceptable daily intake

of clomazone by livestock is 0.12 mg Kg⁻¹ (PPDB 2019), our results show that a slight change in such a concentration (i.e. 0.111 in our study) may still affect other organisms that are more sensitive and that should be taken into account by environmental protection agencies.

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Brenda and Cássia gave technical support to set up and conduct the study. Ítalo contributed with his expertise in plant anatomy and writing the manuscript. Evander gave support in the statistical analysis and with his expertise in weed science. Dayana and Jose Barbosa were the supervisors of this research. Victor was in charge of conducting the experiment and writing the manuscript.

