Universidade Federal do Rio Grande
Instituto de Ciências Biológicas
Pós-graduação em Biologia de
Ambientes Aquáticos Continentais

Toxicidade de herbicidas do grupo das Imidazolinonas em *Pontederia crassipes* Mart.

Igor Alexander de Moura Silva

Orientador: Prof. Dr. Junior Borella
Coorientador: Prof. Dr. Marcos Antonio Baccarin

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Aos meus pais,
que mesmo com todas as dificuldades da vida, nunca desistiram.
Vocês sempre me inspiraram a correr atrás dos meus sonhos.
Palavras são pequenas para expressar o tamanho do meu amor e gratidão.

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Quem decide ir atrás dos seus sonhos raramente encontra um percurso fácil. Inúmeras questões podem tentar te desviar do seu foco e, às vezes, você pode se perder da trilha que vai te levar até lá. Me considero vitorioso por chegar até aqui, porque sei que foi um caminho longo, um pouco tortuoso, mas de MUITO aprendizado. E como a felicidade não é completa, se ela não for compartilhada, deixo aqui minha imensa gratidão a quem esteve do meu lado e contribuiu de alguma forma para alcançar esse objetivo.

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Igor Moura

RESUMO

A contaminação de ambientes aquáticos por herbicidas pode afetar organismos não alvo que desempenham papeis essenciais nesses ecossistemas, tais como macrófitas. As imidazolinonas, amplamente aplicadas no cultivo de arroz irrigado no Rio Grande do Sul desde a implementação do sistema Clearfield®, apresentam características (alta solubilidade e persistência) que promovem essa contaminação. Os herbicidas imidazolinonas inibem a enzima acetolactato sintase, interferindo na biossíntese de aminoácidos de cadeia ramificada (valina, leucina e isoleucina), levando à redução da biossíntese de proteínas e DNA, resultando na diminuição da divisão celular e na taxa de crescimento das plantas. No entanto, outros efeitos fisiológicos e bioquímicos também são frequentemente observados. O objetivo desse trabalho foi avaliar a toxicidade de herbicidas imidazolinonas em macrófitas, utilizando formulação comercial (imazapir + imazapique) e *Pontederia crassipes* Mart. (aguapé) como modelo biológico. Os efeitos do herbicida foram testados em dois experimentos distintos: no Experimento I foi avaliado a toxicidade visual das doses de 0.2, 0.5 e 1.0 mg L^{-1} e tratamento controle; no Experimento II foi avaliado efeitos bioquímicos e fisiológicos da dose 0.5 mg L^{-1} e tratamento controle. As plantas apresentaram sintomas de clorose e necrose, e tiveram o desenvolvimento de biomassa interrompido nas três doses utilizadas no experimento I. No experimento II, as plantas apresentaram insuficiência fotoquímica, prejudicando a assimilação de carbono e fotorrespiração. O herbicida também provocou o fechamento estomático, resultando em redução da condutância estomática e aumento da concentração intracelular de CO_2. Esse efeito foi acompanhado por sintomas de estresse oxidativo e alterações no sistema de defesa observados tanto nas folhas quanto nas raízes. No entanto, ao contrário do esperado, as folhas maduras analisadas apresentaram um aumento na atividade da ALS após o tratamento com o herbicida. Esse resultado inesperado pode sugerir um mecanismo compensatório, onde o aumento da atividade da ALS nas folhas maduras poderia ser uma resposta à supressão da ALS nos tecidos novos. Assim, considerando os papéis vitais que *P. crassipes* desempenham ecossistemas aquáticos, a contaminação desses ambientes por IMI derivados das áreas de cultivo de arroz pode ter efeitos adversos em cadeia, afetando a biodiversidade, a estrutura do habitat e a qualidade da água.

Palavras-chave: contaminação; estresse oxidativo; fitotoxicidade; fotossíntese; macrófita aquática.

ABSTRACT

The contamination of aquatic environments by herbicides can affect non-target organisms that play essential roles in these ecosystems, such as macrophytes. Imidazolinones, widely applied in rice cultivation in Rio Grande do Sul since the implementation of the Clearfield® system, possess characteristics (high solubility and persistence) that promote this contamination. Imidazolinone herbicides inhibit the enzyme acetolactate synthase, interfering with the biosynthesis of branched-chain amino acids (valine, leucine, and isoleucine), leading to a reduction in protein and DNA biosynthesis, resulting in decreased cell division and plant growth rates. However, other physiological and biochemical effects are also frequently observed. The objective of this study was to evaluate the toxicity of imidazolinone herbicides on macrophytes, using a commercial formulation (imazapyr + imazapic) and *Pontederia crassipes* Mart. (water hyacinth) as a biological model. The herbicide's effects were tested in two distinct experiments: Experiment I evaluated the visual toxicity of doses 0.2, 0.5, and 1.0 mg L^{-1} and a control treatment; Experiment II evaluated the biochemical and physiological effects of the 0.5 mg L^{-1} dose and a control treatment. The plants showed symptoms of chlorosis and necrosis and had biomass development interrupted at all three doses used in Experiment I. In Experiment II, the plants exhibited insufficient photosynthetic efficiency, impairing carbon assimilation and photorespiration. The herbicide also induced stomatal closure, leading to reduced stomatal conductance and increased intracellular CO_2 concentration. This effect was accompanied by symptoms of oxidative stress and alterations in the defense system observed in both leaves and roots. However, contrary to expectations, the mature leaves analyzed showed an increase in ALS activity following herbicide treatment. This unexpected result may suggest a compensatory mechanism, where the increase in ALS activity in mature leaves could be a response to ALS suppression in new tissues. Given the vital roles that *P. crassipes* plays in aquatic ecosystems, contamination of these environments by IMI herbicides from rice cultivation areas could have adverse cascading effects, impacting biodiversity, habitat structure, and water quality.

Keywords: contamination; oxidative stress; phytotoxicity; photosynthesis; aquatic macrophyte.

APRESENTAÇÃO

Esse trabalho foi dividido em três partes: Introdução Geral, Capítulo 1 e Considerações finais. A formatação seguida foi de acordo com as normas da ABNT, exceto pelo capítulo 1, em que a formatação segue as recomendações da revista *Environmental Science and Pollution Research* em que o trabalho será submetido. A introdução geral contém uma breve revisão sobre contaminação de recursos hídricos por pesticidas, uma caracterização dos recursos hídricos e agricultura no Rio Grande do Sul. Ainda na introdução, as Imidazolinas e efeitos do mecanismo de ação dos herbicidas são caracterizadas, e *Pontederia crassipes* é apresentada como modelo biológico. Já o capítulo 1, está organizado no formato de manuscrito intitulado "*Physiological and biochemical responses of Pontederia crassipes to Imidazolinones herbicides-group exposure*".

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INTRODUÇÃO GERAL

Os pesticidas têm sido identificados como uma das principais fontes de contaminação de ambientes aquáticos (Della-Flora et al., 2019; Reiber et al., 2020). Consequentemente, esses ambientes destacam-se entre os recursos naturais mais afetados por esses produtos, recebendo uma quantidade significativa de resíduos provenientes de áreas agrícolas (Back; Deschamps; Santos, 2016; Mottes et al., 2017; Sandin et al., 2018), principalmente através de processos de drenagem, lixiviação, escoamento superficial e subsuperficial, erosão e deriva (Gavrilescu, 2005; Triegel; Guo, 2018).

Estudos apontam a presença de pesticidas em diferentes recursos hídricos, como fontes de água subterrânea (Guzzella; Pozzoni; Giuliano, 2006) e superficiais (Kronbauer et al. 2021; Moreira et al., 2012), bem como o risco de contaminação da água para abastecimento público (Gasparini; Vieira, 2010). Um exemplo é a detecção do herbicida clomazone em água para consumo humano, presente em mais de 50% das amostras em quatro anos de monitoramento no sul do Brasil (Caldas et al., 2019).

De acordo com Kronbauer et al. (2021), essa contaminação de águas superficiais e subterrâneas é facilitada pela chuva, que leva ao deslocamento dos pesticidas das áreas cultivadas para rios, poços, riachos e córregos. Além disso, as características das moléculas e das formulações dos produtos utilizados nessas produções também influenciam o potencial de contaminação, devido aos comportamentos de mobilidade, solubilidade e persistência das moléculas no solo e/ou na água (Salazar-Ledesma et al., 2018). Pesticidas com alta solubilidade e persistência representam um maior risco de contaminação das águas superficiais e subterrâneas (Passos et al., 2019; Silva et al., 2019). Além disso, a degradação das moléculas de alguns pesticidas pode ser longa, podendo persistir nesses ecossistemas aquáticos por anos (Börjesson et al., 2004; Grützmacher, et al., 2008).

Nesse sentido, a contaminação de ambientes aquáticos por pesticidas provenientes do cultivo podem afetar a qualidade da água (Pericherla; Karnena; Vara, 2020), afetar importantes serviços ambientais que esses ecossistemas desenvolvem (Spadotto, 2006; Berti et al. 2009;), causar a mortalidade de peixes, interferindo na estrutura das comunidades ao atingir espécies não alvo (Miron et al., 2005) e afetar também organismos produtores que desempenham um papel vital nesses ambientes, tais como macrófitas (IUPAC, 2019).

Recursos hídricos e agricultura no Rio Grande do Sul

O Rio Grande do Sul (RS) possui uma riqueza de ecossistemas aquáticos de grande importância ambiental e econômica, sendo considerado privilegiado por sua alta disponibilidade de águas superficiais (Basso, 2012). Essa riqueza de recursos hídricos favoreceu o desenvolvimento da agricultura no estado, uma vez que essas produções demandam de um grande volume de água para irrigação.

A Lei Estadual 10.350/1994 determina a existência de três Regiões Hidrográficas no RS: a Região da Bacia do Guaíba, a Região das Bacias Costeiras e a Região do Rio Uruguai (CEVS/SES, 2010). O Decreto 53.885, de 18 de janeiro de 2018, estabelece a subdivisão das Regiões Hidrográficas em 25 Bacias Hidrográficas, das quais grandes volumes de água são derivados para a irrigação dessas culturas (Basso, 2012).

Entre as culturas no RS (Fig. 1A), destaca-se o cultivo de arroz (*Oryza sativa* L.) (Fig. 1B), que representa cerca de 71% de toda a produção nacional (CONAB, 2022). Essas produções são de grande importância econômica e tem forte influência no PIB (produto interno bruto) do estado, servindo como principal fonte de renda para aproximadamente 129 municípios que tem como base o cultivo de arroz irrigado (SOSBAI, 2018).

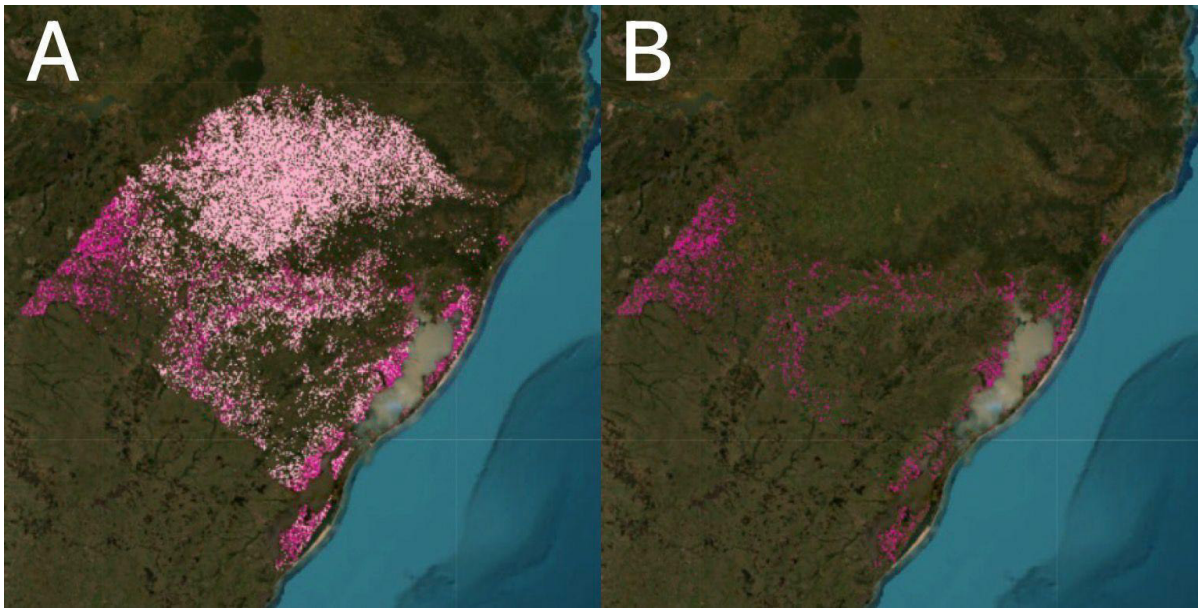

Figura 1: Regiões agrícolas no Estado do Rio Grande do Sul (pontos rosas). Todas as culturas, tanto temporárias quanto perenes (A), e o cultivo de arroz (B).

Fonte: MapBiomas (2022)

Para assegurar essa produtividade, a aplicação de herbicidas para o controle de plantas daninhas é fortemente presente, uma vez que a infestação é um fator limitante na produção de arroz (Ziska, 2016). Os herbicidas mais comumente utilizados no cultivo de arroz incluem Bentazona, Bispiribaque-sódico, Clomazona, Cialofope-butílico, 2,4-D dimetilamina, Etoxissulfurom, Glifosato, Only, Kifix, Ally, Oxifluorfem, Pendimetalina, Penoxsulam e Propanil (Sosbai, 2018).

No entanto, apesar da eficiência no controle de plantas daninhas que prejudicam a produtividade do arroz, o uso de herbicidas pode resultar em contaminação dos recursos hídricos de grande importância no estado. Esses compostos podem ser transportados para ambientes aquáticos próximos aos cultivos através de processos de dispersão ou através dos próprios efluentes da irrigação descarregados nos corpos d'água (Machado et al., 2006). Um exemplo, é a presença de uma série de ingredientes ativos de herbicidas detectados em mananciais hídricos de 19 regiões orizícolas no RS, com maiores concentrações durante e logo após o fim do ciclo do cultivo de arroz irrigado (Silva et al., 2009).

O manejo de plantas daninhas foi otimizado com a introdução do sistema Clearfield® (CL), caracterizado pelo uso de arroz portador de genes tolerantes a herbicidas do grupo químico das Imidazolinonas. O sistema CL foi desenvolvido através da indução de mutações com etil metanossulfonato (EMS), resultando na substituição do códon GGG por GAG na posição 654 (domínio E da ALS) (Alves, 2019). De acordo com Wetzel et al. (1999), uma única substituição na base nucleotídica da ALS impede que o herbicida se ligue à enzima. Portanto, ao aplicar o herbicida nos cultivos de arroz CL, esses não são afetados, uma vez que o sítio de ligação da ALS resistente não os reconhece.

No Brasil, vários outros cultivares resistentes foram desenvolvidos, como AVAXI CL, BRS A701 CL, BRS Sinuelo CL, GURI INTA CL, INOV CL, IRGA 422 CL, IRGA 428 CL, IRGAP H7CL, IRGAP H9 CL, LEXUS CL, PUITÁ INTA CL, SCS 117CL, SCS121 CL, TITAN CL e 33 IRGA 424 CL (SOSBAI, 2018). No Rio Grande do Sul, o sistema CL foi implementado na safra 2003/2004 e representa cerca de 80% das plantações de arroz do estado (EMBRAPA, 2022).

Imidazolinonas

As imidazolinonas (IMI) são um grupo de herbicidas sintéticos descobertos na década de 1980, e incluem o *Imazametabenz-metílico* [metil 2-(4-isopropil-4-metil-5-oxo-2-imidazolin-2-il)-p-toluato + metil 6-(4-isopropil-4-metil-5-oxo-2-imidazolin-2-il)-m-toluato

(3:2)], *Imazamox* [ácido 2-(4-isopropil-4-metil-5-oxo-2-imidazolin-2-il)-5-(metoximetil) nicotínico], *Imazapic* [ácido 2-(4-isopropil-4-metil-5-oxo-2-imidazolin-2-il)-5-metilnicotínico], *Imazapyr* [ácido 2-(4-isopropil-4-metil-5-oxo-2-imidazolin-2-il) nicotínico], *Imazaquin* [ácido 2-(4-isopropil-4-metil-5-oxo-2-imidazolin-2-il)-3-quinolínico] e *Imazetapir* [ácido 5-etil-2-(4-isopropil-4-metil-5-oxo-2-imidazolin-2-il) nicotínico] (Hess et al., 2010). Junto das sulfoniluréias, triazolopirimidinas, pirimidiloxitiobenzoatos e sulfonilamino-carboniltriazolinonas, as IMI fazem parte da classe de herbicidas inibidores da enzima Acetolactato Sintase [ALS; também referida como Acetohidroxiácido Sintase (AHAS)] (Zhou et al., 2007), essencial na via metabólica de biossíntese de aminoácidos alifáticos de cadeia ramificada (valina, leucina e isoleucina) nas plantas (Tan et al., 2005).

A ALS catalisa duas reações: 1) a condensação de duas moléculas de piruvato para formar acetolactato, precursor de valina e leucina; e 2) condensação de piruvato + cetobutirato para formar acetohidroxiácido, precursor de isoleucina (Fig. 2) (Vargas et al., 2016). A inibição da ALS por herbicidas do grupo IMI é dita não competitiva, pois sua ligação não ocorre no sítio catalítico de seus substratos (Chang; Duggleby, 1997). No entanto, ao se ligar à enzima por meio de outro domínio de ligação, as IMI impedem a ligação com o piruvato (McCourty et al., 2006), interferindo na via metabólica desses aminoácidos. A deficiência desses aminoácidos, por sua vez, leva à diminuição na síntese de proteínas e DNA, prejudicando os processos de divisão celular e translocação de fotoassimilados (Shaner; Singh, 1993).

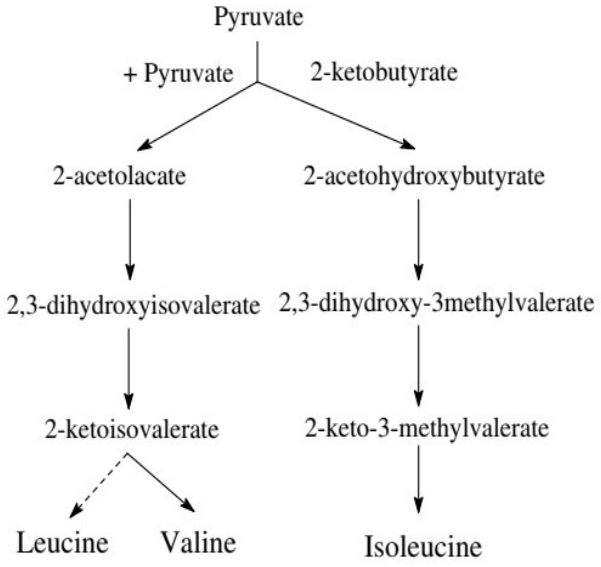

Figura 2: Resumo da rota metabólica para a síntese dos aminoácidos valina, leucina e isoleucina.

Fonte: Zhou et al. (2007)

Devido a ALS ser mais expressa e importante em tecidos meristemáticos, como pontos de crescimento e tecidos reprodutivos, essas regiões são os primeiros a demonstrarem sintomas de toxicidade (Singh; Shaner, 1995). De acordo com Jursik et al. (2010), as IMI são transportadas tanto pelo xilema quanto pelo floema até o local-alvo, onde imediatamente bloqueiam a divisão celular. Assim, alguns dias após aplicação, os tecidos meristemáticos morrem primeiro, seguidos por necrose lenta dos tecidos maduros (Shaner; Anderson; Stidham, 1984).

Além disso, autores apontam o sintoma de clorose em diferentes plantas expostas as IMI (Cruz et al., 2015; Oliveira Jr, 2011; Piveta et al., 2018; Sala et al., 2008; Shaner, 2017), mesmo que seu mecanismo de ação não seja um supressor direto da biossíntese de pigmentos fotossintéticos. Assim, considerando que os pigmentos (clorofila *a*, clorofila *b* e carotenoides) são essenciais para a absorção da energia luminosa usada para desencadear o processo de conversão em energia química na fotossíntese (Meng et al. 2018; Wen et al. 2019), sua diminuição ou ausência pode levar a uma redução na taxa fotossintética (Arief et al., 2023).

Condições que resultam em alterações desses parâmetros fotossintéticos podem levar a diminuição na fixação de CO_2 no metabolismo fotossintético do carbono. Isso porque a energia produzida na cadeia de transporte de elétrons, na forma de ATP e NADPH, são necessárias no ciclo de Calvin-Benson para a formação de esqueletos de carbono (carboidratos) para o desenvolvimento das plantas (Stirbet et al., 2020). Somado a isso, a redução na condutância estomática em resposta ao fechamento estomático, que são estrutura altamente sensíveis a estresses abióticos, como a exposição a herbicidas (Taiz; Zeiger, 2004), pode resultar em um aumento na concentração de CO_2 (Kayoumu et al., 2023; Jones, 1992).

A diminuição no conteúdo de pigmentos devido à atividade de IMI também pode levar ao estresse oxidativo (Fraga et al., 2020). Embora as IMI não sejam os principais indutores de estresse oxidativo, esses sintomas podem ocorrer em plantas expostas a esse grupo de herbicidas (Ochogavía et al., 2014). Nesse sentido, o excesso de energia luminosa não processado eficientemente na fotossíntese pode causar o escape de elétrons da cadeia de transporte de elétrons, resultando em espécies reativas de oxigênio (ROS) (Li; Kim 2022; Van Breusegem et al. 2001), induzindo a peroxidação lipídica da membrana celular, resultando em vazamento incontrolável de eletrólitos e morte celular (Dayan; Watson, 2011).

Diante da exposição a compostos tóxicos como herbicidas e estresse oxidativo, que podem afetar moléculas de DNA, lipídios, proteínas e carboidratos, as células passam por processos bioquímicos para combatê-los (Coleman; Randall; Blake-Kalff, 1997; Sies, 1993). O sistema de defesa antioxidante envolve componentes não enzimáticos como compostos

fenólicos, ácido ascórbico (AsA), glutationa (GSH), β-caroteno e α-tocoferol, e componentes enzimáticos como superóxido dismutase (SOD), ascorbato peroxidase (APX) e enzimas catalase (CAT) (Barbosa et al., 2014). As SODs são metalo-enzimas que defendem contra ERO catalisando a dismutação de dois radicais $O_2^{\bullet-}$ (ânion superóxido) para gerar H_2O_2 (peróxido de hidrogênio) e O_2, reduzindo o risco de formação de OH• (radicais hidroxila), o oxidante mais reativo na família de EROs (Dinakar; Djilianov; Bartels, 2012; Dubey, 2011). Por outro lado, as enzimas APX e CAT são as mais importantes no mecanismo de detoxificação de H_2O_2, uma ERO moderadamente reativa (Bhatt; Tripathi, 2011). Em relação à detoxificação de contaminantes químicos, a enzima glutationa S-transferase (GST) desempenha um papel essencial nesse processo (Hu, 2014).

Outro distúrbio frequentemente observado em plantas expostas as IMI é o acúmulo de carboidrato nas folhas, independente do estágio de desenvolvimento da planta (Armendáriz et al., 2016; Gaston et al., 2002; Scarponi et al., 1996; Zabalza et al., 2004). No entanto, esse aumento não resulta de uma maior produção em plantas tratadas com herbicida, sendo possivelmente originado do amido armazenado, por consequência dos distúrbios na fotossíntese (Smith; Zeeman, 2020). Somado a isso, os inibidores de ALS são apontados como um supressor do transporte de fotoassimilados aos pontos de crescimento, aumentando os níveis nas folhas (Kim; Born, 1997; Zabalza et al., 2004).

Nesse sentido as IMIs são consideradas altamente eficazes e atuam sob um amplo espectro de plantas daninhas, sendo elas de folhas estreitas ou largas (Tan et al., 2005). Assim, quando associadas ao sistema Clearfield, apresentam alta eficiência no combate seletivo de plantas daninhas sem causar perdas nas culturas (Scarabel et al., 2012; SOSBAI, 2016). Dentre os herbicidas IMI, a formulação comercial Kifix® (525g i.a. ha^{-1} de imazapyr + 175g i.a. ha^{-1} de imazapic) é amplamente recomendada pelo sistema CL (SOSBAI, 2016).

No entanto, apesar de sua alta eficiência, apresentam características que favorecem a contaminação de fontes hídricas, uma vez que são altamente solúveis e persistentes no ambiente (Kraemer et al., 2009). Os ingredientes ativos presentes na formulação Kifix, apresentam meia-vida no solo variando de 30 e 133 dias para o Imazapyr (Wang et al., 2005), e de 120 a 140 dias para o Imazapic (Tu; Hurd; Randall, 2001). Já na água, essas variações no período de persistência também são observadas de acordo com as condições locais, como pH e disponibilidade de luz (Kraemer et al., 2009). Enquanto o Imazapic pode ter uma meia-vida curta de apenas 1 ou 2 dias (Tu; Hurd; Randall, 2001), outras condições levaram a persistência de traços de Imazapyr em águas subterrâneas 8 anos após a aplicação (Börjesson et al., 2004). Dessa forma, IMIs são encontradas em diferentes recursos hídricos, como águas subterrâneas e

superficiais (Battaglin et al., 2000; Marchesa et al., 2010; Mazlan; Hussain; Zawawi, 2016; Silva et al., 2009; Tu; Hurd; Randall, 2001).

Sua ocorrência pode afetar espécies de macrófitas aquáticas não-alvo que desempenham um papel vital na manutenção e equilíbrio desses ecossistemas. Macrófitas aquáticas são responsáveis pela qualidade da água, ciclo de nutrientes e fluxo de energia (Esteves, 1988), servem como fonte de alimento e habitat para outros organismos (Rolon; Maltchik, 2006) e são associadas com alta diversidade e produtividade de corpos d'água (Chambers et al., 2008). Diante disso, é necessário compreender como as IMI, amplamente utilizadas no cultivo de arroz irrigado do RS, podem afetar macrófitas aquáticas que habitam regiões próximas as áreas de cultivos, que desempenham papeis essenciais para nesses ecossistemas.

Pontederia crassipes Mart.

Pontederia crassipes Mart. [também conhecida como *Eichhornia crassipes* (Mart.) Solms.] (Fig. 3) é uma planta nativa da América do Sul (Barrett; Forno, 1982) e atualmente está distribuída por todos os estados do Brasil (Sousa, 2020), incluindo o Rio Grande do Sul, devido sua abundância em ambientes aquáticos. A espécie demonstra uma notável capacidade de crescer em diversos tipos de ecossistemas aquáticos, como lagos, riachos, lagoas, canais e valas, tanto em climas frios quanto quentes (Ayanda; Ajayi; Asuwaju, 2020), o que aumenta significativamente a probabilidade de colonização nos ambientes aquáticos próximos às regiões orizícolas.

Popularmente conhecida como aguapé ou jacinto-de-água, *P. crassipes* é uma monocotiledônea aquática da família Pontederiaceae; no entanto, pode sobreviver em ambientes sazonalmente não alagados por curtos períodos devido à sua plasticidade fenotípica (Leandro; Holsback; Scremin-Dias, 2021). Sua forma de vida é fixa ou flutuante, com numerosas raízes com coifas bem desenvolvidas (Pott; Pott, 2000). As folhas são pecioladas, rígidas e com cutícula espessa, com lâminas de formato sagitado ou ovalado e pecíolos inflados, apresentando grandes estômatos tanto na lâmina foliar quanto no pecíolo, que facilitam e contribuem para o processo de transpiração (Kissmann; Roth, 1997); suas flores roxas ou violetas a tornam uma popular planta ornamental (Seema, 2012).

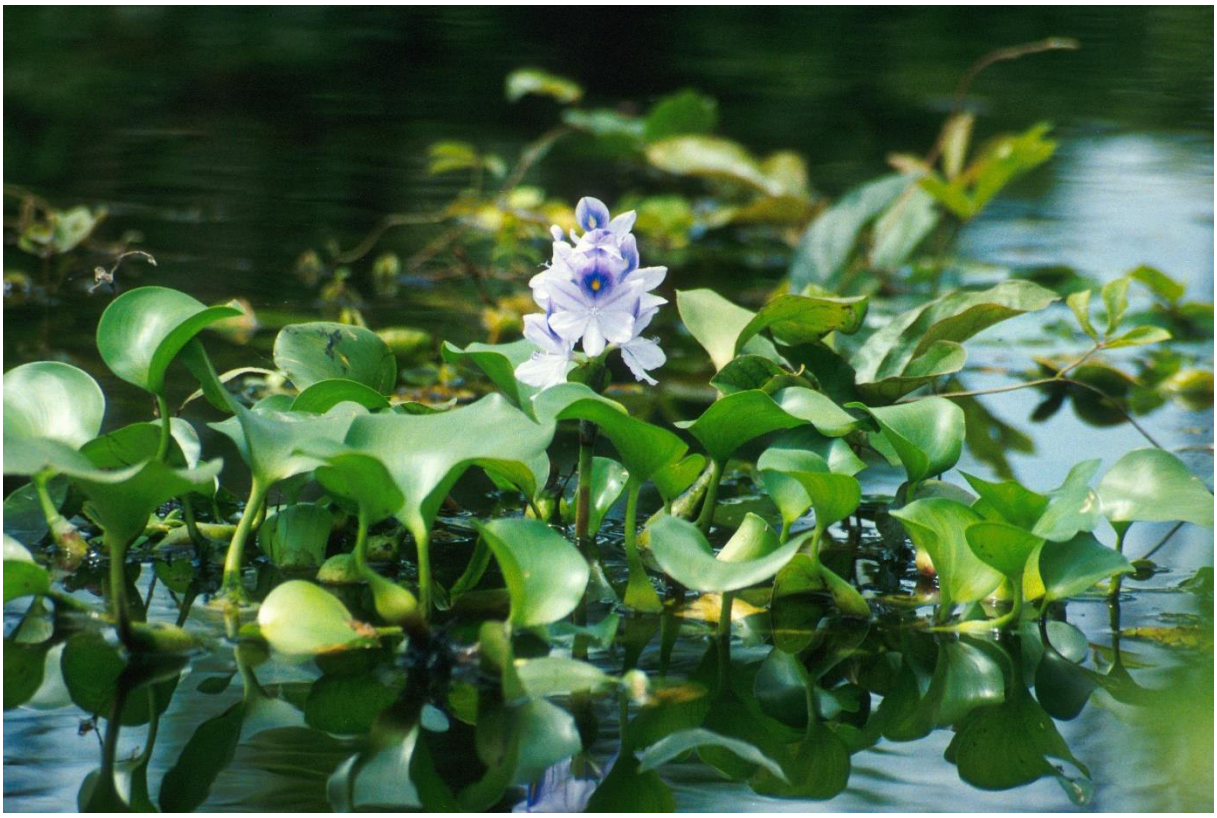

Figure 3: *Pontederia crassipes* (Mart.) Solms. em fase de floração no ambiente aquático.

Fonte: wikimedia.org

Reproduz-se sexualmente por sementes que podem permanecer viáveis por anos, mas sua principal forma de reprodução ocorre por propagação vegetativa a partir de talos que se desenvolvem por brotamento (Gopal, 1987). Segundo Martins et al. (2009), seu potencial reprodutivo é tão grande que pode aumentar sua área de cobertura em até 15% por dia, dobrando-a a cada seis ou sete dias, desde que haja condições ambientalmente favoráveis de matéria orgânica, luz e água. Portanto, devido à sua ampla distribuição, rápida reprodução e desenvolvimento, são bons modelos para análises fitotoxicológicas de exposição aguda ao herbicida proposto pelo presente projeto.

Além disso, *P. crassipes* tem grande capacidade de tolerar ambientes contaminados, uma vez que conseguem modificar sua fisiologia e anatomia, levando a espécie a se adaptar a ambientes estressantes (Pereira, 2010). Em ambientes contaminados com cádmio (Cd), os aguapés apresentaram alterações anatômicas e fisiológicas, como aumento na densidade estomática, espessura do mesófilo foliar e parênquima esponjoso, aumento da taxa de fotossíntese e, consequentemente, o desenvolvimento da planta (Pereira, 2010). Alencar et al. (2020) também observaram, através de sintomas visuais e anatômicos, que *P. crassipes* teve

aproximadamente 58% maior tolerância ao herbicida clomazone do que a espécie *Pistia stratiotes* (Araceae).

Por outro lado, outros estudos demonstram sintomas significativos de toxicidade em *P. crassipes* expostas a herbicidas (Neves; Foloni; Pitelli, 2002; Robles; Madsen; Wersal, 2010; Yoshii, 2022). Costa et al. (2011) identificaram alterações na anatomia das regiões da nervura central e internervural do limbo foliar quando expostos aos herbicidas diquat e 2,4-D, o que poderia afetar a dinâmica de transporte de moléculas através desses vasos condutores. Concentrações de glifosato resultaram na interrupção do crescimento, clorose, necrose e degeneração tecidual de aguapés após a exposição (Maria et al., 2018). Portanto, uma variedade de sintomas pode ser observada em aguapés expostos a diferentes contaminantes e, quando relacionado à exposição a herbicidas, esses sintomas diferem de acordo com o mecanismo de ação do herbicida (Mendes; Silva, 2023).

Portanto, considerando que *P. crassipes* é uma planta capaz de modificar sua fisiologia, bioquímica e anatomia, levando a espécie a se adaptar a condições estressantes como exposição a certos contaminantes, o presente estudo teve como objetivo avaliar o potencial tóxico de herbicidas IMI através da exposição aguda de aguapés à formulação comercial Kifix® (imazapic + imazapyr) através de dois experimento, sendo um para analises de toxicidade visual e outro para demais parâmetros bioquímicos e fisiológicos.

Embora os aguapés exibam plasticidade fenotípica (Leandro; Holsback; Scremin-Dias, 2021), atribuindo-lhes a capacidade de se adaptar a condições estressantes, neste estudo nós testamos a hipótese que *P. crassipes* teriam sintomas de clorose e necrose o seu desenvolvimento seria interrompido após aplicação das doses de 0.2, 0.5 e 1.0 mg L^{-1} da formulação comercial Kifix. Além disso, também levaria a outras interferências negativas, como insuficiência fotossintética, formação de espécies reativas de oxigênio e estresse oxidativo, bem como outros distúrbio fisiológicos e bioquímicos avaliados após aplicação da dose de 0.5 mg L^{-1} de Kifix.

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CAPÍTULO 1

O manuscrito segue a formatação solicitada pela revista *Environmental Science and Pollution Research*, a qual esse trabalho será submetido. No entanto, para facilitar a visualização, as imagens foram adicionadas ao longo do texto.

Physiological and biochemical responses of *Pontederia crassipes* to Imidazolinones herbicides-group exposure

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Abstract

The imidazolinone herbicides, inhibitors of the acetolactate-synthase enzyme, interfere with the biosynthesis of branched-chain amino acids, leading to reduced protein and DNA synthesis, resulting in decreased cell division and relative growth rate. However, other secondary disturbances can occur in plants treated with these herbicides. The aim of this work was to evaluate physiological and biochemical effects caused by a commercial formulation (imazapyr + imazapic) beyond the directly effects of the mode of action on *Pontederia crassipes*. These effects were assessed in two separate experiments: Experiment I with doses of 0.2, 0.5, and 1.0 mg L^{-1} plus an untreated control; Experiment II with 0.5 mg L^{-1} and untreated control. The plants exhibited symptoms of chlorosis and necrosis, and their biomass development was halted at all three doses used in Experiment I. In Experiment II, the treated plants exhibited photochemical insufficiency, impairing carbon assimilation and photorespiration. The herbicide also caused stomatal closure, reducing conductance and increasing intracellular CO_2 concentration. Leaves and roots showed symptoms of oxidative stress and alterations in the defense system. However, contrary to expectations, the mature leaves analyzed demonstrated increased ALS activity after herbicide treatment, which may indicate a compensatory mechanism for the suppression of ALS in the new tissues of *P. crassipes*

Keywords contamination; fluorescence; oxidative stress; phytotoxicity; photosynthesis; aquatic macrophyte

Introduction

The State of Rio Grande do Sul (RS) stands out as the largest producer of rice (*Oryza sativa* L.) in Brazil (CONAB 2024). To increase rice production, technologies and management improvements have been introduced, such as the Clearfield® (CL) system, characterized by the use of rice plants carrying genes tolerant to herbicides from the imidazolinone (IMI) chemical group. The CL system was implemented in the 2003/2004 season and represents approximately 80% of the cultivated area (EMBRAPA 2022). The IMI operates by inhibiting Acetolactate synthase [ALS; also referred to as Acetohydroxyacid synthase (AHAS)], which is an essential enzyme in the metabolic pathway for the biosynthesis of branched-chain aliphatic amino acids (valine, leucine and isoleucine) (Tan et al. 2005). This inhibition leads to decreased protein and DNA synthesis in meristematic tissues,

disrupting cell division and the transport of photoassimilates to growth points in a wide diversity of weed species (Zhou et al. 2006).

Despite the highly efficiency in selective weed control in CL rice cultivation, IMI have characteristics that favor the contamination of water sources, as they are highly soluble and persistent in the environment (Kraemer et al. 2009). The commercial formulation Kifix® (imazapyr + imazapic), one of the IMI herbicides recommended by the CL system for rice cultivation, shows a half-life ranging from 30 to 133 days for imazapyr and 120 to 140 days for imazapic in soil (Tu et al. 2001; Wang et al. 2005). The residual soil persistence of imazapyr and imazapic shows toxic effects on non-tolerant rice up to two years after the application (Reffati et al., 2017). In water, this period also varies according to environmental conditions, such as pH and light availability (Kraemer et al. 2009). While Imazapic can have a short half-life of only 1 or 2 days (Tu; Hurd; Randall, 2001), other conditions have led to the persistence of traces of Imazapyr in groundwater 8 years after application (Börjesson et al., 2004). In this context, they can reach aquatic ecosystems near the cultivation through dispersion processes or by the irrigation effluents from flooded rice fields being released into water bodies (Machado et al. 2006), affecting non-target plants.

In wetlands of Southern Brazil, the native macrophyte *Pontederia crassipes* Mart. are highly notable due to its abundance of aquatic environments. *P. crassipes* thrives in freshwater bodies near agricultural fields, making it susceptible to contamination from herbicides used in nearby cultivation areas. They also are widely used in phytotoxicity analysis (Costa et al. 2011; Maria et al. 2018), being considered a great biological model due to its wide distribution, rapid reproduction and development (Martins et al. 2009; Sousa 2020).

It has been reported that aquatic macrophytes exposed to IMI demonstrate a decrease in growth parameters, such as biomass of shoot and roots (Emerine et al. 2010; Li et al. 2022; Jablonski et al. 2023). However, other effects not direct caused by the mode of action has been observed, such as chlorosis in *Pontederia crassipes* (Robles et al. 2010), *Pistia stratiotes* (Cruz et al. 2015), *Lemna minor* (Li et al. 2022), and *Salvinia molesta* (Garlich et al. 2021), even the mode of action of IMI are not direct suppressor of pigment biosynthesis.

The chlorosis leads to a significant reduction of the photosynthetic process in macrophytes (Costa et al., 2018). Changes are detected in the chlorophyll (chl) *a* fluorescence in response to photosynthetic disturbances in plants treated with IMI herbicides, such as electron transport through the photosystems, decreasing nearly entirely the performance indices (Sousa et al. 2014). Conditions that result in changes to these photosynthetic parameters can lead to a decrease in CO_2 fixation in plants as a consequence of low ATP and NADPH production (Stirbet et al. 2020). The herbicide exposure can also trigger stomatal closure, which in addition to decreasing assimilation rate, can result in an increase in intracellular CO_2 concentration (Jones 1992; Kayoumu et al. 2023).

The decrease in pigment content due to the activity of imazapyr + imazapic can also cause an oxidative stress (Fraga et al., 2020). Thus, excess light energy can harm the photosynthetic apparatus and lead to the escape of electrons from the electron transport chain, resulting in reactive oxygen species (ROS) (Li and Kim 2022; Van Breusegem et al. 2001). To neutralize the cytotoxic effects of ROS, cells rely on an antioxidant enzyme system (Huang et al. 2019), to avoid interference in damage to DNA and proteins, lipid peroxidation of membranes, electrolyte leakage, and ultimately the cell death (Dayan and Watson 2011; Gill and Tuteja 2010; Halliwell and Gutteridge 2015; Zhao et al. 2017). Glutathione S-transferase (GST) is another important enzyme in the plant defense system, which acts in protection against chemical contaminants by conjugating them with reduced glutathione, facilitating the neutralization of these toxic compounds (Strange et al. 2000; Severo et al. 2020).

Therefore, there are several possible secondary effects yet poorly described that the herbicides imazapyr + imazapic may cause on the metabolism and physiology of non-target aquatic plants. Although IMI herbicides are often described as safe when used at low concentration levels (Hoseiny-Rad and Aivazi 2020; Kumar et al. 2024), several studies describe their toxicity to different aquatic plants (Li et al. 2022; Qian et al. 2009; Wersal and Madsen 2007). Thus, this work aims to evaluate the effects of different doses (0.2, 0.5, and 1.0 mg L^{-1}) of the imazapyr + imazapic formulation on the growth parameters and visual symptoms in P. crassipes, as well as the effects of the 0.5 mg L^{-1} dose on other biochemical and physiological parameters, such as photosynthetic efficiency, gas exchange, oxidative stress and defense system.

Material and methods

Plant material and growth conditions

P. crassipes were manually collected from a small lake located in Pelotas, RS-Brazil (31°44'39" S, 52°19'52" W). The plants were cleaned with running water and individually relocated into pots (4 L) containing 3.5 L of culture solution, comprised of water and half-strength Hoagland nutrient solution (Arnon, 1950). The plants were acclimated in a greenhouse, under natural light conditions of 1500 ± 100 µmol fótons m^{-2} s^{-1} and a temperature of 28 ± 5 °C. The Hoagland solution was replaced every 15 d during the acclimation period and one day before herbicide exposure. Subsequently, plants with similar fresh biomass and size (± 40 g/plant) were selected and standardized by removing all plantlets and old leaves, leaving only the five newly fully expanded leaves. The IMI commercial formulation used was Kifix® [imazapyr (525 g Kg^{-1}; 52.5% w/w) + imazapic (175 g Kg^{-1}; 17.5 % w/w) + a mixture based on sodium alkyl naphthalene sulfonate (37.50 g Kg^{-1}; 3.75% w/w) and other undisclosed ingredients in the label (262.50 g Kg^{-1}; 26.25 % w/w)] (BASF, 2010), with direct application in the water.

Two simultaneous experiments were conducted as described: Experiment I, focused on visual symptoms, chl content, and growth parameters. The experiment was carried out over a period of 14 d with five replicates. Treatments was based on the literature from other studies that evaluated the effects of herbicides on aquatic plants (Ribeiro et al., 2019; Alencar et al., 2020; Santos et al., 2022), consisting of following doses of Kifix: 0.2 mg L^{-1} (105 µg L^{-1} imazapyr + 35 µg L^{-1} imazapic); 0.5 mg L^{-1} (263 µg L^{-1} imazapyr + 88 µg L^{-1} imazapic); 1.0 mg L^{-1} (525 µg L^{-1} imazapyr + 175 µg L^{-1} imazapic), plus one untreated control (0 mg L^{-1}). The Experiment II, focused on additional biochemical and physiological parameters, was also conducted over a 14-day period with five replicates. The treatments consisted of Kifix at 0.5 mg L^{-1} (263 µg L^{-1} imazapyr + 88 µg L^{-1} imazapic), plus an untreated control (0 mg L^{-1}).

Experiment I - Visual symptoms, chl index and growth parameters

Visual toxicity symptoms and chl index was obtained at 7 and 14 d after herbicide application (DAA). Chlorosis and necrosis symptoms in the plant shoot were estimated visually and assigned notes, considering a scale from 0 to 100%. In this scale, 0% represents the absence of the symptom (chlorosis or necrosis) and 100% represents the symptom appearing throughout the shoot (SBCPD 1995). The chl index was estimated using a

portable chl meter, model CL-01 (Hansatech Instruments, King's Lynn, Norfolk, UK), at the central region of the leaf blades.

The plant growth parameters were obtained considering the initial (0 DAA) and final measurements (14 DDA). The number of plantlets and leaf were counted, the total fresh biomass as well as per organ (shoot, root and plantlet) were measured and used to calculate relative growth rate of biomass. To estimate the leaf area of each plant, we considered the correction factor (f) of 0.783 obtained by Rodrigues et al. (2024). The relative growth rate of leaf area was determined by the initial and final measurement of leaf blades of each plant.

Experiment II – Physiological and biochemical analysis

Chl *a* fluorescence transients' measurements

Chl *a* fluorescence analysis was conducted at 7 and 14 DAA using the Handy-Pea portable fluorimeter (Hansatech Instruments Ltd, UK) on the last fully expanded leaf determined at the herbicide application time. Four regions of these leaves were dark-adapted with leaf clips for 30 min before measurements. Chl *a* fluorescence was induced by a saturating red-light flash (peak at 650 nm) with 3.000 μmol photons m^{-2} s^{-1}. For the measurements, the initial fluorescence intensity (F_0 – minimum fluorescence; 50 μs) and the highest (F_M - maximum fluorescence) and their intermediates were considered during 1 s of illumination (Strasser et al., 2004; Tsimilli-Michael and Strasser, 2008). The Test-JIP parameters and OJIP curves were calculated according to Strasser and Strasser (1995) and Strasser et al. (2004). Additionally, we analyzed the kinetic difference of the relative variable fluorescence (Yusuf et al. 2010).

Gas exchange

Analyses was conducted at 14 DAA, between 9:00 and 10:00 AM on the central region of last fully expanded leaves. Evaluation was conducted using infra-red gas analyzer (IRGA LI-6400XT, LI-COR Inc., Lincoln, NE, USA), with an internal CO_2 concentration of 400 μmol CO_2 mol^{-1}, a photon flux density of 1.500 μmol m^{-2} s^{-1}, and a block temperature of 25 °C. The parameters of CO_2 assimilation (net photosynthesis - *A*), intracellular CO_2 concentration (C_i), stomatal conductance (g_s), and transpiration rate (*E*) were calculated following the model described by Von Caemmerer and Farquhar (1981).

Leaf epidermal anatomy

Samples was collected from the right side of the apical portion of the leaf blades at 14 DAA. The samples were fixed in FAA 50% (formaldehyde, acetic acid and 50% ethanol; 1:1:18, v/v) for 72 h and subsequently stored in 70% ethanol until the analyses were performed. The leaf blade epidermis was dissociated using Franklin's solution (Johansen 1940) and stained with 1% safranin (Kraus and Arduim 1997). Histological slides of the adaxial and abaxial epidermal surfaces of the leaf blades were prepared in a glycerol-water mixture (1:1, v/v), on a semi-permanent slide. These slides were observed under an optical microscope (Olympus BX53) equipped with a digital camera (Olympus DP72) for image capture. The ANATI QUANTI software, version 2.0 for Windows® calibrated with a microscopic ruler (Aguiar et al., 2007), was used to manually quantify open and closed stomata, analyze the stomatal index (SI) according to Cutter (1986), and determine stomatal density according to Castro et al. (2009).

Reactive oxygen species levels and lipid peroxidation

Samples of approximately 100 mg of leaves and roots was collected at 14 DDA. The generation rate of $O_2^{\bullet-}$ was determined according to Li et al. (2010). Samples were ground in liquid nitrogen, homogenized with a 65 mM potassium phosphate buffer (pH 7.8), and then centrifuged at 5.000 *g* at 4 °C for 10 min. The supernatant was mixed with 65 mM potassium phosphate buffer (pH 7.8) and 10 mM hydroxylamine were added and incubated at 25 °C for 20 min. After incubation, 17 mM sulfanilamide and 7 mM α-naphthylamine were added, followed by another incubation at 25 °C for 20 min. Absorbance was measured at 530 nm, using a standard curve of known sodium nitrite concentrations. The H_2O_2 level was determined according to Velikova et al. (2000). Samples were macerated in liquid nitrogen, homogenized with 0.1% trichloroacetic acid (TCA), and then centrifuged at 12.000 *g* at 4 °C for 20 min. Potassium phosphate buffer 10 mM (pH 7.0) and potassium iodide were added to the extract for H_2O_2 content analysis. Absorbance was measured at 390 nm using a standard curve of known H_2O_2 concentrations.

Lipid peroxidation was determined using the TBARS (Thiobarbituric Acid Reactive Substances) method by Cakmak and Horst (1991). The supernatant obtained from the procedure conducted for the H_2O_2 content was added to a reaction medium containing 0.5% thiobarbituric acid (TBA) in 10% (w/v) TCA to quantify malondialdehyde (MDA), a product generated during H_2O_2 decomposition. The mixture was heated at 90 °C in a water bath for 20 min, then cooled in an ice bath to reach room temperature. After centrifugation at 10.000 g for 5 min, absorbance readings were taken at 535 and 600 nm using a spectrophotometer to detect the red compound generated in the reaction MDA-TBA. The molar extinction coefficient value (ε_{535nm} = 155 x 10^{-3} M^{-1} cm^{-1}) was used to calculate the concentration of the MDA-TBA complex and express lipid peroxidation.

Electrolyte leakage

Samples of approximately 100 mg of leaves and roots was collected at 14 DDA. The electrolyte leakage was determined according to Lima et al. (2002). Samples were immersed in deionized water at room temperature for 1 h. Initial conductivity (Ci) was measured using a conductivity meter (Hanna Instruments HI2300). Subsequently, the samples were then incubated in a water bath at 90 °C for an additional 1 hour, and the final conductivity (Cf) was measured. The data for initial conductivity (Ci) and final conductivity (Cf) were expressed as a percentage using the equation described by Tarhanen et al. (1999).

Antioxidant enzymes activity

Samples of approximately 100 mg of leaves and roots was collected at 14 DDA for analysis of the activities of superoxide dismutase (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6), and ascorbate peroxidase (APX: EC 1.11.1.11). Samples were macerated in liquid nitrogen and 5% polyvinylpolypyrrolidone (PVPP). They were then homogenized in extraction buffer containing 100 mM potassium phosphate (pH 7.8), 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM sodium ascorbate. After centrifugation at 12.000 g for 20 min at 4 °C, the supernatant was collected for enzyme activity assessment. SOD activity was determined according to Giannopolitis and Ries (1977), by measuring its ability to inhibit the photoreduction of nitrotetrazolium blue (NBT) at 560 nm. The reaction mixture included the supernatant, 50 mM potassium phosphate buffer (pH 7.8), 14 mM methionine, 0.1 µM EDTA, 75 µM NBT and 2 µM riboflavin. CAT activity was determined according to Azevedo Neto et al. (2006), by monitoring the oxidation of H_2O_2. The reaction mixture contained the supernatant, 100 mM potassium phosphate buffer (pH 7.0) and 12.5 mM hydrogen peroxide. The oxidation of H_2O_2 was

measured by monitoring the decrease in absorbance at 240 nm at 10 s intervals over a 2 min period. APX activity was determined based on the oxidation of sodium ascorbate according to Nakato and Asada (1981). The reaction mixture included the supernatant, 100 mM potassium phosphate buffer (pH 7.0), 0.5 mM sodium ascorbate and 0.1 mM hydrogen peroxide. The oxidation of sodium ascorbate was determined by monitoring the decrease in absorbance at 290 nm at 10 s intervals over a 2 min period.

Glutathione S-transferase activity

Samples of approximately 100 mg of leaves and roots was collected at 14 DDA to determinate the activities of glutathione S-transferase (GST, EC 2.5.1.13). Samples were macerated in liquid nitrogen and homogenized in an extraction buffer containing 200 mM Phosphate buffer (pH 7.8), 0.1 mM EDTA and 5 mM β-mercaptoethanol. After centrifugation at 12.000 g for 20 min at 4 °C, the supernatant was collected for GST activity assessment. GST activity was determined following Dalton et al. (1993), using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. The collected supernatant was mixed with 100 mM potassium phosphate buffer (pH 7.5), 1 mM glutathione (GSH), and 1 mM CDNB for the reaction, which was incubated for 2 min. The reaction was monitored by measuring absorbance at 340 nm at 10 s intervals over a 2 min period. The specific activity of GST was determined by the formation of the GS-DNB conjugate ($\varepsilon 340nm = 6.2 \times 10^3\ M^{-1}\ cm^{-1}$) and expressed as μmol mg^{-1} of protein min^{-1}. Protein quantification was performed using the methodology described by Bradford (1976) from the collected supernatant.

Glycolate oxidase activity

Samples of approximately 100 mg of leaves was collected at 14 DDA to determinate the activities of glycolate oxidase (GO, EC 1.1.3.1). Samples were macerated in liquid nitrogen with 5% polyvinylpolypyrrolidone (PVPP), then homogenized in a 100 mM Tris-HCl buffer at pH 7.8 (containing 0.01% Triton X-100 and 5 mM dithiothreitol). After centrifugation at 12.000 g for 15 min at 4 °C, the supernatant was collected for GO activity assessment. GO activity was determined according to Bai et al. (2014), where the supernatant was added to a reaction mixture containing 50 mM Tris-HCl buffer (pH 7.8), 0.009% Triton X-100 (v/v), 3.3 mM phenylhydrazine-HCl (pH 6.8), plant extract and 5 mM glycolate (pH 7.0). The reactions were monitored by the formation of phenylhydrazone glyoxylate ($Ɛ324nm = 17\ mM^{-1}\ cm^{-1}$) at 30 °C and absorbance was measured at 324 nm at 10 s intervals over a 2 min period.

Acetolactate synthase activity

Samples of approximately 100 mg of leaves and roots was collected at 14 DDA to determinate the activities of acetolactate synthase (ALS, EC 4.1.3.18). Samples were macerated in liquid nitrogen and homogenized in an extraction buffer containing 100 mM potassium phosphate (pH 7.5), 0.5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate (TPP), 10 uM flavin adenine dinucleotide (FAD), 10 mM sodium pyruvate, 10% v/v glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.5% soluble PVP. After centrifugation at 12.000 g for 15 min, the supernatant was employed in a reaction mixture containing 50 mM HEPES, pH 7.5, 200 mM sodium pyruvate, 20 mM $MgCl_2$, 2 mM TPP, and 20 uM FAD and incubated at 37 °C for 60 min. To stop the reaction, 6 N H_2SO_4 were added and incubated at 60 °C for 15 min to convert acetolactate to acetoin. Then, 0.55% creatine solution and 5,5% of a-naphthol solution in 5 N NaOH were added and incubated

at 60 °C for 15 min. The ALS activity was determined according to Yu et al. (2010), by measuring the formation of acetoin with absorbance 530 nm.

Carbohydrates, amino acids and protein content

Samples of approximately 100 mg of leaves and roots was collected at 14 DDA to determinate the total soluble sugars (TSS), sucrose, amino acids and protein content. TSS, sucrose and total amino acids content were extracted following the methodology outlined by Bieleski and Turner (1966) with modifications. Samples were macerated with liquid nitrogen and homogenized with 20 mL g^{-1} of fresh weight of methanol:chloroform:water (12:5:3 v/v). The extracts were centrifuged after 24 h at 600 g for 30 min. For every 4 mL of supernatant, 1.0 mL of chloroform and 1.5 mL of water were added, followed by another centrifugation to separate the phases. The supernatant was then collected and transferred to a water bath at 38 ºC for 30 h to remove the chloroform residue and concentrate the samples for analyses. The precipitate obtained from the initial centrifugation was resuspended in 0.1 N sodium hydroxide to obtain the protein fraction. After 24 h, the extracts were centrifuged at 2.000 g for 30 min and the supernatant used for protein quantification.

The TSS determination was conducted as described by Graham and Smydzuk (1965), using a concentrations series of glucose for quantification, while sucrose was determined according to Handel (1968), using a concentration series of sucrose for quantification. The amount of total amino acids was determined using the ninhydrin method of Yemm and Cocking (1955) using a concentration series of leucine for quantification. The protein content was determined using Bradford solution (Bradforf 1976), using a concentration series of bovine serum albumin for quantification.

Statistical analysis

Two separate experiments were conducted employing a completely randomized design. Each experiment featured one plant per pot, which served as the experimental unit each one with five replicates per treatment. The data were subjected to tests of normality (Shapiro–Wilk) and homogeneity. Subsequently, statistical analysis was performed using one-way analysis of variance (ANOVA) for the experiment I. When significant differences were detected via the *F*-test in ANOVA, means among herbicide concentrations were compared using the Tukey test at a significance level of $p \leq 0.05$. The experiment II compared using the t-test at a significance level of $p \leq 0.05$. The statistical analyses were carried out using SAS 8.0 statistical software program (SAS Institute Inc., Cary, NC, USA). Furthermore, the data were exported to the MetaboAnalyst web-based platform (http://www.metaboanalyst.ca/) for additional analysis. Filters based on standard deviations were applied solely for auto-scaling the data. The data were subjected to Principal Component Analysis (PCA) to evaluate segregation among treatments in each experiment.

Results

Experiment I

Visual Symptoms

Plants treated with the three doses of commercial herbicide (0.2, 0.5, and 1.0 mg L^{-1}) exhibited visual symptoms of chlorosis and necrosis (Fig. 1A-C). Additionally, 14 days after herbicide application (DAA), it was

observed that bud (plantlet) growth was interrupted in the 0.2, 0.5, and 1.0 mg L^{-1} treatments (Fig. 1D). The symptom assessments revealed a progression of chlorosis in the 0.5 and 1.0 mg L^{-1} treatments, with a significant increase between the analyses conducted at 7 and 14 DAA (Fig. 1E). In these same treatments (0.5 and 1.0 mg L^{-1}), there was a significant decrease in the total chlorophyll index between 7 and 14 DAA (Fig. 1G). Necrotic spots appeared on the leaf blades of treated plants at 14 DAA, absent in the 7 DAA analysis (Fig. 1F).

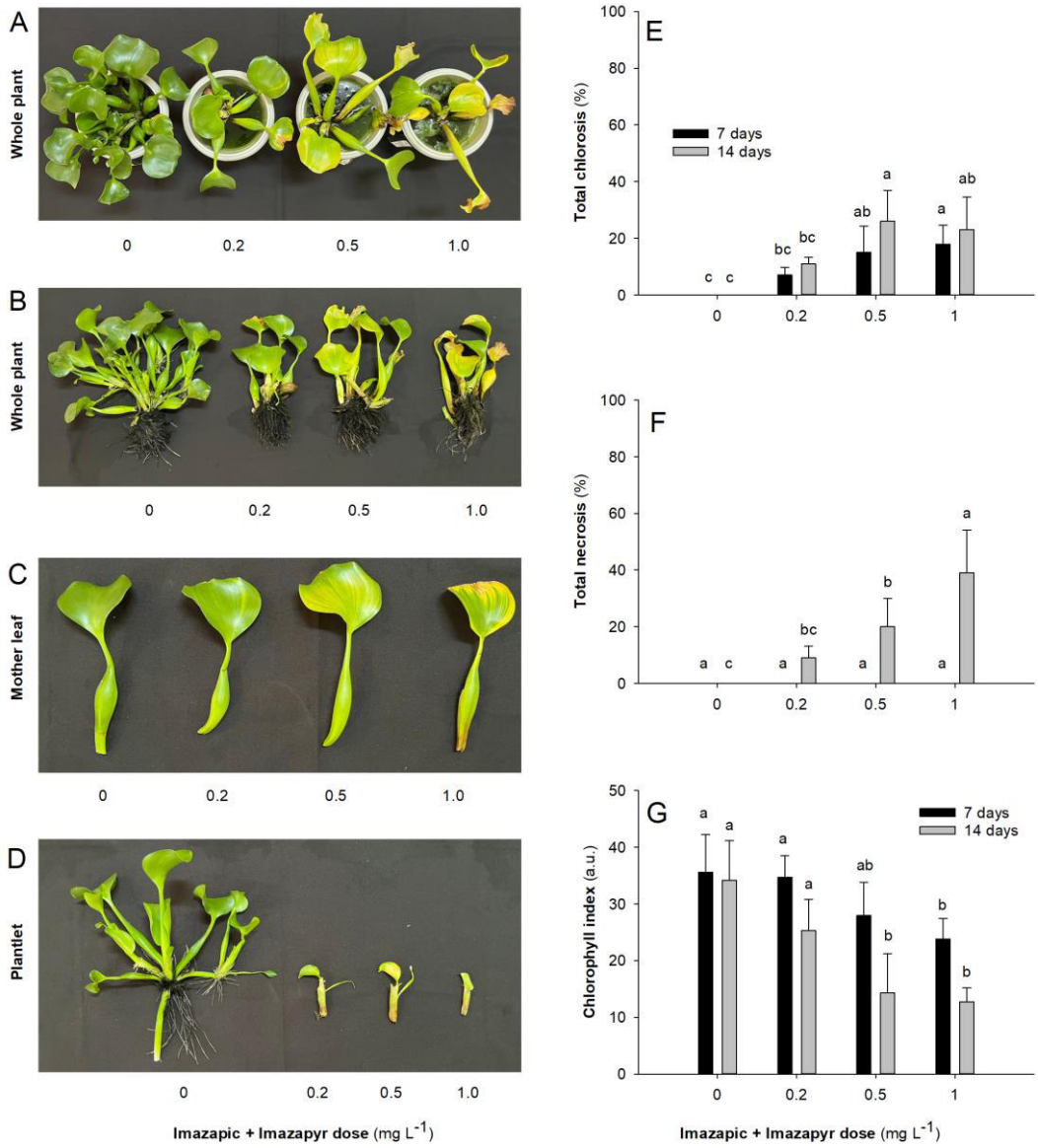

Fig. 1. Visual symptoms in *Pontederia crassipes* treated with different commercial doses of imazapic + imazapyr herbicide. Whole shoot plant (A); Whole plant (B); Last fully expanded leaves exposed to herbicide (C); Plantlets developed after herbicide exposure (D); Total chlorosis (E); Total necrosis (F); Chlorophyll index (G). The values are presented as the mean ± standard deviation (SD). Means followed by the same letter do not differ among the treatments (herbicide doses), according to the Tukey test ($P \leq 0.05$, $n = 5$).

Growth and Biomass Parameters

The total fresh biomass of untreated plants (0 mg L^{-1}) increased (2-fold higher), while the treated plants did not show any increase in biomass (Fig. 2A). The greatest biomass development of untreated plants occurred in the plantlets and leaves, while in the treated plants was severely affected, as evidenced by the weighing of separated organs (Fig. 2B) and the number of leaves and shoots developed after herbicide application (Fig. 2D). The treated plants showed even a negative relative growth rate, indicating biomass loss (Fig. 2C). The leaves of untreated plants showed a significant increase in leaf area compared to the leaves of treated plants (Fig. 2E). The RGR of leaf area also showed negative values in the 1.0 mg L^{-1} treatment (Fig. 2F).

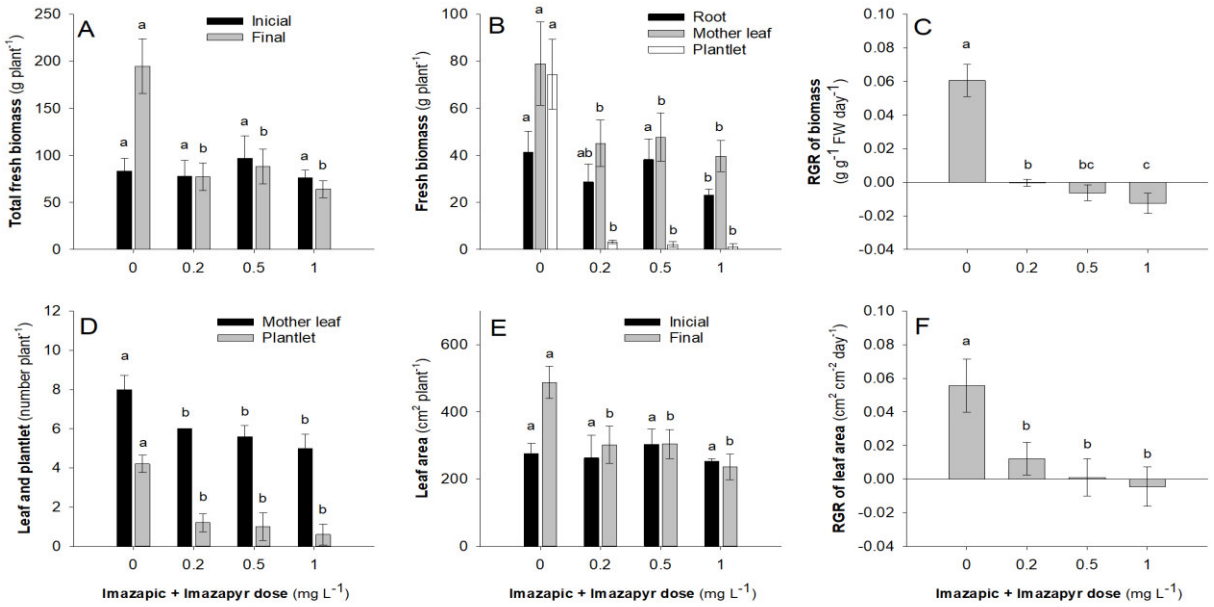

Figure 2. Growth parameters of *Pontederia crassipes* treated with different commercial doses of imazapic + imazapyr herbicide. Total fresh biomass (A); Root, leaf, and shoot fresh biomass (B); Relative growth rate of biomass (C); Number of leaves and shoots (D); Leaf area (E); Relative growth rate of leaf area (F). The values are presented as the mean ± standard deviation (SD). Means followed by the same letter do not differ among the treatments (herbicide doses), according to the Tukey test ($P \leq 0.05$, $n = 5$).

Experiment II

Chlorophyll *a* fluorescence

All plants exhibited the characteristic chlorophyll transient curves (W_t) observed between initial fluorescence (0.05 ms) and maximum fluorescence (1 s). However, in treated plants was observed an increase in fluorescence intensity at steps J (2 ms) and I (30 ms) compared to the untreated plants (Fig. 3C). Furthermore, after normalizing the O and I step, treated plants showed a slightly increase in the sequence of events, from exciton capture by photosystem II (PSII) to plastoquinone reduction (W_{OI}) (Fig. 3D). The treated plants also showed an increase in the curve and a decrease in the events of W_{IP} and W_{OI} >1, respectively (Fig. 3E). This indicates that

the sequence of events between the electron transfer from PQH_2 (plastoquinol) to the final electron acceptors on the PSI acceptor side had a drastic decrease after 14 d of herbicide treatment.

Additional normalizations and corresponding subtractions [$\Delta W_{OJ} = W_{OJ(herbicide)} - W_{OJ(untreated)}$; $\Delta W_{OK} = W_{OK(herbicide)} - W_{OK(untreated)}$] revealed the presence of the K-band between steps O and J (Fig. 3F) and the L-band between steps O and K (Fig. 3G) in herbicide-exposed plants. The positive L-band, highlighted after normalization, represents lower energy connectivity between PSII and I, indicating inefficient processing of excitation energy and decreased system stability. In contrast, the positive K-band reflects an increase in antenna complex and/or reduction in the oxygen-evolving complex (OEC).

The interference in the photosynthetic machinery of treated plants was more pronounced at 14 DAA (Fig. 3A and B). The OEC of treated plants showed a significant decrease, indicating the inability of a portion of the reaction centers to transport electrons. However, there was a significant increase in light absorption by the reaction center (ABS/RC), energy dissipation in the form of heat (DI_0/RC) and energy capture by the reaction center (TR_0/RC). The electron transport by the reaction center (ET_0/RC) showed no difference compare to untreated group. The Maximum quantum yield of primary photochemistry (ϕ_{Po}) was slightly affected. However, steps in the electron transport chain after the reduction of quinone A were more affected, as can be seen in decrease of Efficiency/probability for electron transport (ET), i.e. efficiency/probability that an electron moves further than quinone A (QA^-) (ψ_{Eo}), Quantum yield for ET (ϕ_{Eo}), Efficiency/probability with which an electron from the intersystem electron carriers moves to reduce end electron acceptors at the PSI acceptor side (RE) (δ_{Ro}) and Quantum yield for RE (ϕ_{Ro}). Thus, at 14 DAA the treated plants showed a strong decrease in performances indexes, such as Performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors (PI_{abs}) and to the reduction of PSI end acceptors (PI_{total}) (Fig. 3B).

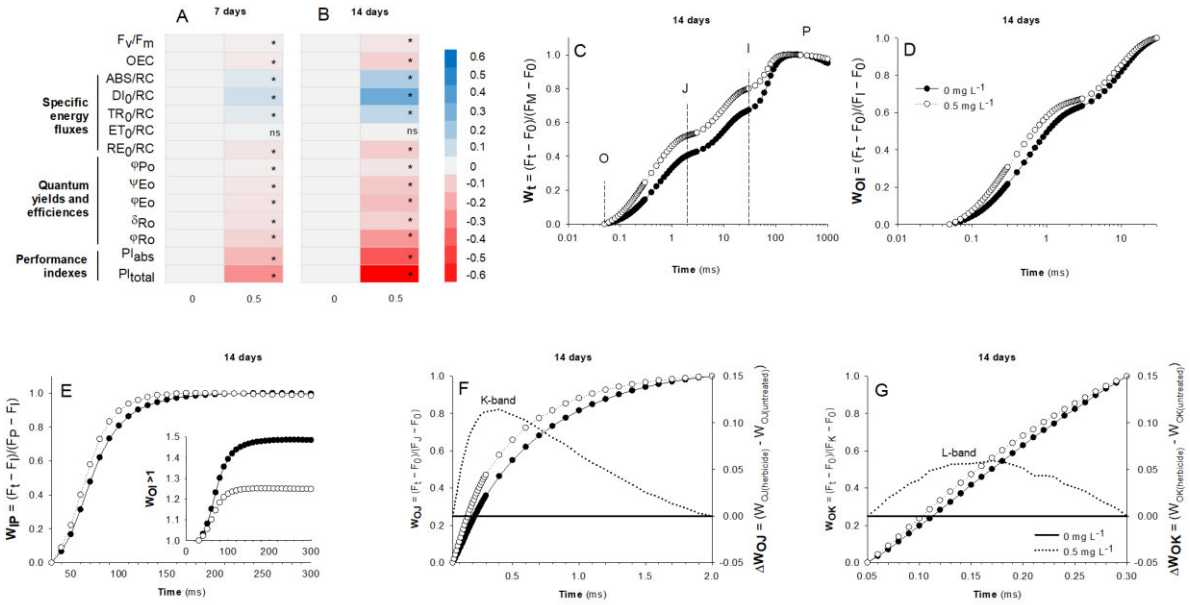

Figure 3. Chlorophyll *a* fluorescence parameter in leaves of *Pontederia crassipes* treated with different commercial doses of imazapic + imazapyr herbicide. Heatmap of biophysical parameters after 7 (A) and 14 (B) days of exposure; Relative variable fluorescence between steps O and P (W_t) in logarithmic time scale (C); relative variable fluorescence between steps O and I (W_{OI}) on logarithmic time scale (D); relative variable fluorescence between steps I and P (W_{IP}) on logarithmic time scale and W_{OI} >1 insertion (E); average kinetics between steps O

and J (W_{OJ}) (F); and between steps O and K (W_{Ok}) (G). Heatmap (A and B): Plotted as $\log_{10}$= (herbicide/untreated), where blue indicate an increase and red a decrease in relation to the untreated group. Means (represented by colors) followed by asterisk (*) indicate difference and followed by "ns" indicate no difference between the treatments (herbicide doses) according to the t test ($P \leq 0.05$, n = 5). F_v/F_m: Ratio between variable fluorescence and maximum fluorescence - quantum efficiency of PSII; OEC: Oxygen-evolving complex; ABS/RC: Absorption flux (of antenna Chls) per reaction center (RC) or effective antenna size of an active RC; DI_0/RC: Dissipation of an active RC; TR_0/RC: Maximum trapping flux (leading to Q_A reduction) per RC; ET_0/RC: Electron transport flux (further than Q_A^-) per RC; RE_0/RC: Electron flux reducing end electron acceptors at the PSI acceptor side, per RC; ϕ_{Po}: Maximum quantum yield of primary photochemistry; ψ_{Eo}: Efficiency/probability for electron transport (ET), i.e. efficiency/probability that an electron moves further than quinone A (Q_A^-); ϕ_{Eo}: quantum yield for electron transport (ET); δ_{Ro}: Efficiency/probability with which an electron from the intersystem electron carriers moves to reduce end electron acceptors at the PSI acceptor side (RE); ϕ_{Ro}: Quantum yield for reduction of end electron acceptors at the PSI acceptor side (RE); PI_{abs}: performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors; PI_{total}, performance index (potential) for energy conservation from exciton to the reduction of PSI end acceptors.

Gas Exchange and leaf epidermis anatomy

The treated plants showed a drastic decrease in Net CO_2 assimilation (*A*), Stomatal conductance (g_s) and transpiration rate (*E*) (Fig. 4A, C and D). An increase in Intracellular CO_2 concentration (C_i) was observed (Fig. 4B). The stomatal index and density on both adaxial and abaxial leaf surfaces showed no difference between treated and untreated plants (Fig. 4F and G). However, treated plants had interference in their stomatal opening on both the adaxial and abaxial surfaces, with 80% and 70% of the stomata closed, respectively (Fig. 4E, F, and G).

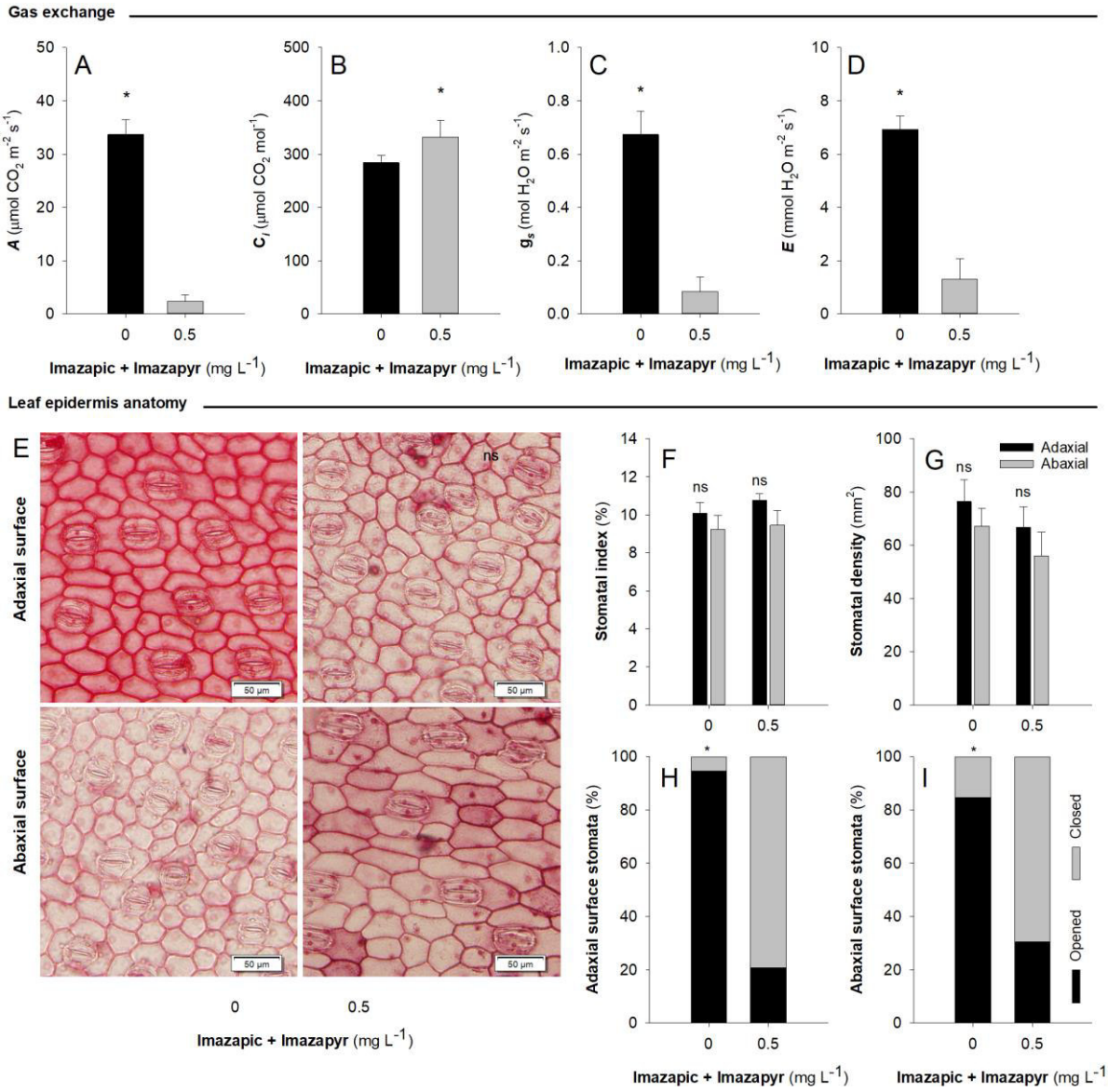

Figure 4. Gas exchange and leaf epidermis anatomy of *Pontederia crassipes* treated with different commercial doses of imazapic + imazapyr herbicide. CO_2 net assimilation rate (*A*) (A); Intracellular CO_2 concentration (C_i) (B); Stomatal conductance (g_s) (C); Transpiration rate (*E*) (D); Stomata on the adaxial and abaxial surfaces (E); Stomatal index (F); Stomatal density (G); Adaxial surface stomata (H); Abaxial surface stomata (I). The values are presented as the mean ± standard deviation (SD). Means followed by an asterisk (*) indicates differences between the treatments (herbicide doses) by t test ($P \leq 0.05$, n = 5). ns = not significative.

Carbohydrates content

Treated plants showed an increase in both total soluble sugars and sucrose in the leaves (Fig. 5A and B). However, in the roots, there were no difference compared to the untreated plants.

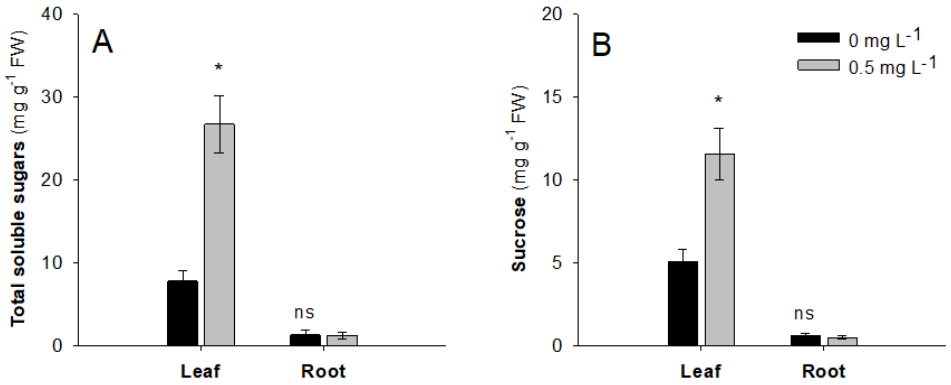

Figure 5. Carbohydrates content in *Pontederia crassipes* treated with different commercial doses of imazapic + imazapyr herbicide. Total soluble sugar (A) and sucrose (B). The values are presented as the mean ± standard deviation (SD). Means followed by an asterisk (*) indicates differences between the treatments (herbicide doses) by t test ($P \leq 0.05$, n = 5). ns = not significative.

Oxidative stress in leaves and roots

Leaves of treated plants showed a lower superoxide generation rate (Fig. 6A) and higher hydrogen peroxide (Fig. 6B) compared to the untreated plants. Roots show no difference in both reactive oxygen species. Additionally, leaves of treated plants showed higher lipid peroxidation (Fig. 6C) and roots showed significant increase of electrolyte leakage (Fig. 6D).

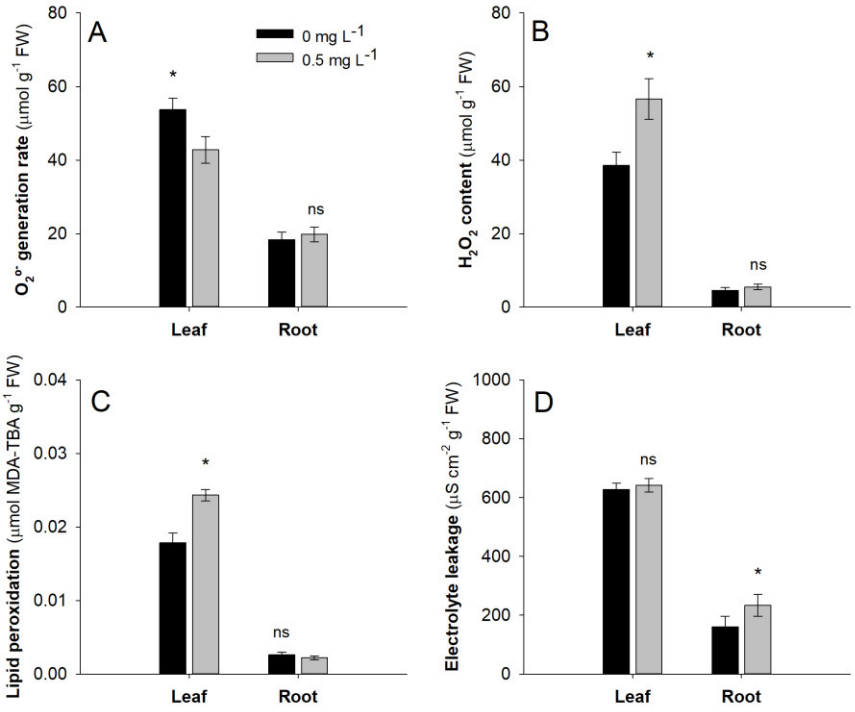

Figure 6. Oxidative stress in the leaves and roots of *Pontederia crassipes* treated with different commercial doses of imazapic + imazapyr herbicide. Superoxide levels (A); Hydrogen peroxide levels (B); Lipid peroxidation (C); Electrolyte leakage (D). The values are presented as the mean ± standard deviation (SD). Means followed by an

asterisk (*) indicates differences between the treatments (herbicide doses) by t test ($P \leq 0.05$, n = 5). ns = not significative.

Antioxidant enzymes, GST and GO activities

Leaves of treated plants showed a decrease in SOD, CAT, GST and GO activity (Fig. 7A, B, D and E) and no difference in APX activity (Fig. 7C). In roots CAT, APX and GST activity increased in treated plants.

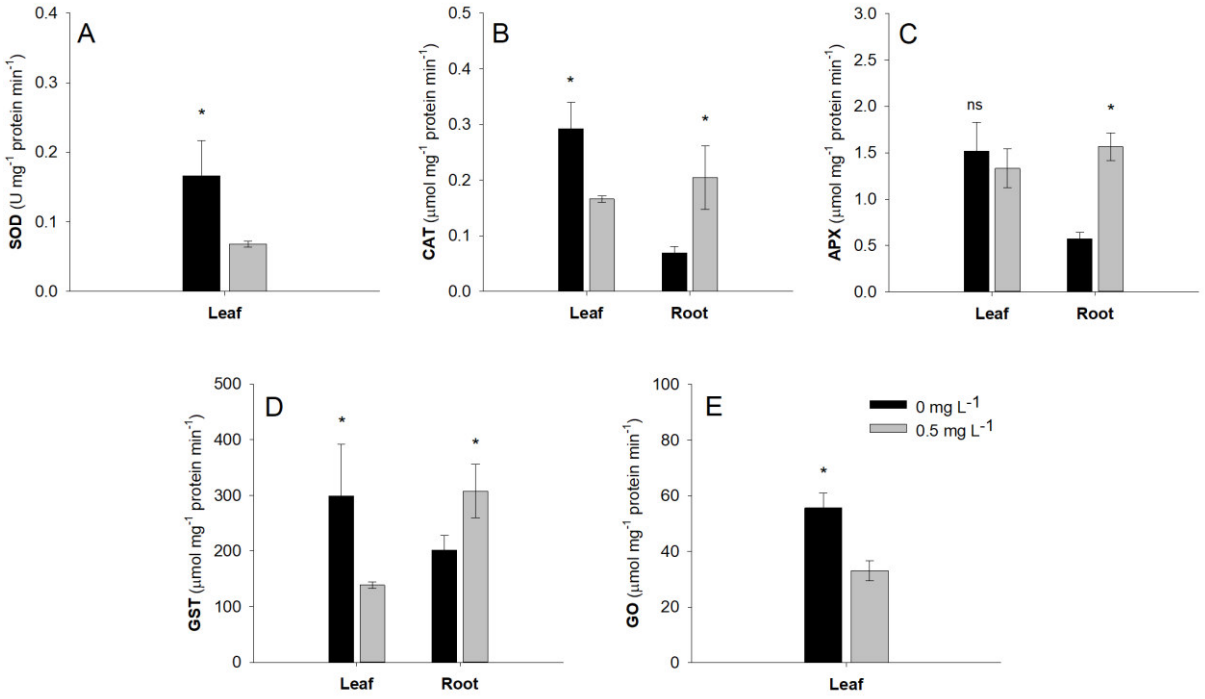

Figure 7. Antioxidant enzyme, glutathione S-transferase and glycolate oxidase activity in *Pontederia crassipes* treated with different commercial doses of imazapic + imazapyr herbicide. Superoxide dismutase activity in leaves; (A); Catalase activity in leaves and roots (B); Ascorbate peroxidase activity in leaves and roots (C); Glutathione S-transferase activity in leaves and roots (D); Glycolate oxidase activity in leaves (E). The values are presented as the mean ± standard deviation (SD). Means followed by an asterisk (*) indicates differences between the treatments (herbicide doses) by t test ($P \leq 0.05$, $n = 5$). ns = not significative.

ALS activity, total amino acids, and protein content.

Treated plants showed an increase in ALS activity in leaves and no difference in roots compare to untreated plants (Fig. 8A). An increase of total amino acid content was observed in the roots (Fig. 8B). The protein content did not undergo changes in either the roots or the leaves in treated plants (Fig. 8C).

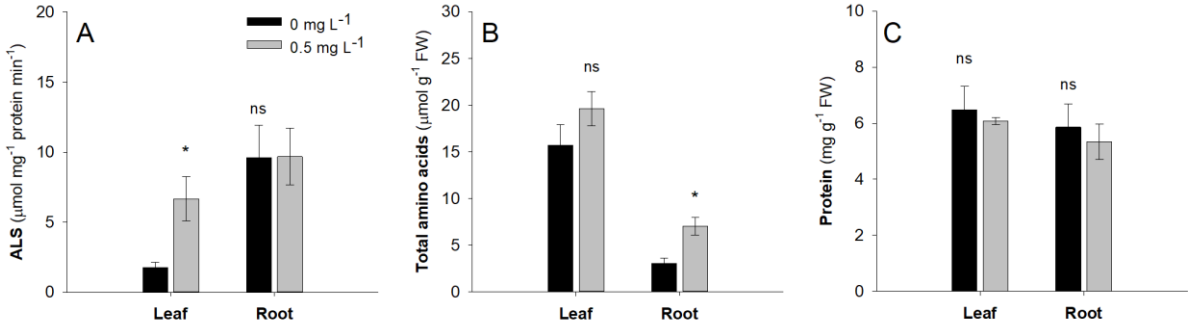

Figure 8. Activity of acetolactate synthase (A), total amino acids (B), and protein (C) content in leaves and roots of *Pontederia crassipes* treated with different commercial doses of imazapic + imazapyr herbicide. The values are presented as the mean ± standard deviation (SD). Means followed by an asterisk (*) indicates differences between the treatments (herbicide doses) by t test ($P \leq 0.05$, n = 5). ns = not significative.

The principal component analysis

The principal component analysis (PCA) allowed the visualization of group formation based on responses of *P. crassipes* after herbicide treatment. The PCA analyses were split between visual symptoms and growth parameters in Experiment I, and biochemical and physiological parameters, as well as chl *a* fluorescence, evaluated in experiment II. Regarding visual and growth responses, the PCA showed segregation of the treatments, with one group comprising the untreated group and the other grouping the plants treated with 0.2, 0.5 and 1.0 mg L^{-1} (Fig. 3A). From physiological and biochemical analyses, the PCA also showed significant segregation between treatments (Fig. 3B). In chl *a* fluorescence parameter, the 0 and 0.5 mg L^{-1} groups overlapped, indicating that for some evaluated parameters, the difference between them was low, not separating them into strongly distinct groups.

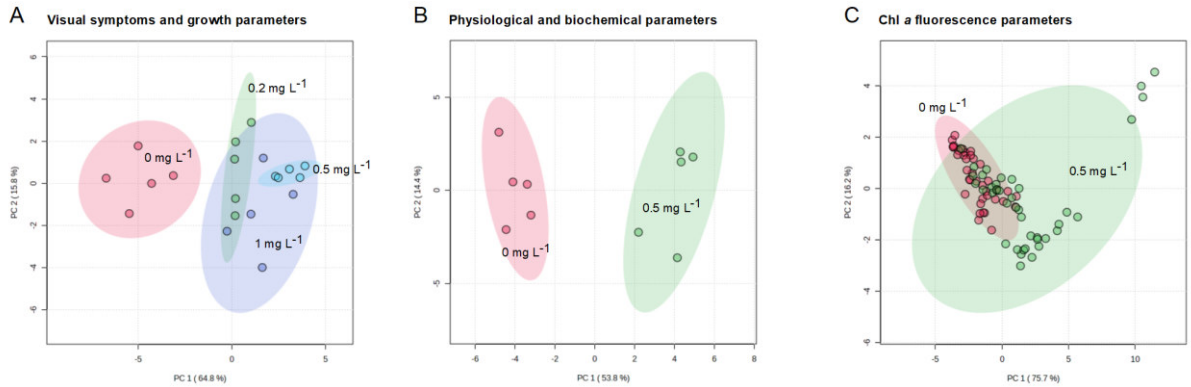

Figure 9. Cluster analysis by principal component analysis (PCA) of *Pontederia crassipes*. plants subjected to different commercial doses of imazapic + imazapyr herbicide. Visual symptoms and growth parameters (A), physiological and biochemical parameters (B) and chlorophyll *a* fluorescence parameter (C).

Discussion

Pontederia crassipes is extensively distributed across aquatic ecosystems in Rio Grande do Sul, rendering it vulnerable to the toxic effects of IMI herbicides originating from irrigated rice cultivation. Upon absorption and translocation to the target sites, IMIs inhibit the biosynthesis of branched-chain aliphatic amino acids (BCAAs), resulting in disruptions in protein and DNA synthesis, both of which are critical for growth (Tan et al., 2005; Villa et al., 2006). As a result, macrophytes exposed to these herbicides exhibit a significant reduction in growth rates within a short period (Cruz et al., 2015; Wersal & Madsen, 2007). In Experiment I, all three tested doses (0.2, 0.5, and 1.0 mg L^{-1}) induced visible symptoms, including chlorosis and necrosis, and adversely impacted the development of new tissues, including the emergence of new leaves, plantlet formation, and reductions in relative growth rates of biomass and leaf area. Beyond impairing the development of new tissues, the results from Experiment II demonstrated that the herbicide also exerts additional physiological and biochemical effects on mature tissues. These effects include photosynthetic inefficiency, elevated intracellular CO_2 concentration due to stomatal closure, increased production of reactive oxygen species (ROS), and oxidative stress, evidenced by lipid peroxidation in leaves and electrolyte leakage in roots. Furthermore, we also observed an increase in ALS activity in mature leaves suggesting a potential compensatory mechanism for the suppression of ALS at growth points.

In Experiment I, chlorosis was observed in all plants exposed to IMI herbicides, as anticipated. Numerous studies have demonstrated that plants treated with various doses of these herbicides exhibit yellowing of leaves due to a reduction in chl content, despite IMI not directly targeting the metabolic pathway of photosynthetic pigments (Cruz et al., 2015; Ochogavía et al., 2014; Sala et al., 2008; Shaner, 2017). The observed decline in chl index following IMI exposure is likely a consequence of photooxidation (Cayon et al., 1990; Duke, 1985) and impaired biosynthesis of new pigments during the experiment due to disruptions in protein metabolism caused by the herbicide's mode of action (Alonge, 2000). In contrast, necrosis was not evident until the final visual assessment at 14 days after application (DAA), suggesting that tissue death progresses more slowly and becomes apparent only after an extended period post-treatment.

Our results demonstrated the herbicide's substantial impact on the growth of *P. crassipes*. Untreated plants experienced a doubling of biomass throughout the experiment period. In contrast, all treated plants, regardless of the dose, exhibited negative values for relative biomass growth rate, indicating not only growth inhibition but also a loss of fresh biomass due to necrotic lesions. This growth disruption can be attributed to impaired cell division (Pillmore and Caseley, 1987; Jursik et al., 2010) and deficiencies in carbohydrate transport to the growth points (Shaner, 2017; Villa et al., 2006). Additionally, because the IMI herbicides are translocated to the growth points, disrupting the development of meristematic tissues, the formation of new leaves and plantlets was significantly affected, with disruptions observable even at the initial stages. Given that vegetative growth is the primary reproductive mechanism for *P. crassipes* (Cardoso et al., 2002), the interference of IMI herbicides with plantlet growth poses a serious threat to the species.

Given the critical role of photosynthetic pigments in capturing light energy for the conversion to chemical energy during photosynthesis (Meng et al., 2018; Wen et al., 2019), the observed reduction in chl content in all treated plants is expected to adversely affect their photosynthetic processes. To further elucidate the impact of herbicide exposure on photosynthesis, we conducted chl *a* fluorescence analysis. This method provides insights into energy absorption and electron transport from Photosystem II (PSII) to the final acceptors in Photosystem I (PSI) (Tsimilli Michael and Strasser, 2008; Yusuf et al., 2010).

In Experiment II, treated plants showed a significative increase in the absorption flux capacity (ABS/RC) per reaction center (RC), indicating that the RCs remaining active were able to maintain some degree of efficiency in sustaining photon flow through the antenna pigments. This enhancement led to a rise in the maximum trapping flux (TR_0/RC), reflecting continued excitation of chl *a* in the active RCs despite a significant decline in chl indexes at 14 DAA. However, the parameter indicating electron transport flux beyond Quinone A (QA^-) per RC (ET_0/RC) did not exhibit significant changes compared to untreated plants. This suggests that, although treated plants showed increased ABS and trapping in the active RCs, the majority of this energy could not proceed through the normal electron transport chain (ETC). This is evidenced by a reduction in the electron flux leading to the reduction of end electron acceptors at the PSI acceptor side (RE_0/RC).

According to Yusuf et al. (2010), an increase in ABS/RC reflects an increase in the apparent antenna size (total absorption or total chl per active RC). The contradictory result between the reduced chl index and the increased absorption can be attributed to the fact that chl *a* fluorescence analysis specifically measures the activity of the active RCs. Consequently, the increase in ABS/RC might be due to the inactivation of a portion of RCs (unable to reduce QA) or due to an enlargement of the functional antenna size to enhance absorption efficiency. To elucidate the underlying phenomenon, the parameter TR_0/RC should be considered. Ideally, in the presence of inactive RCs, the increase in TR_0/RC should be proportional to the increase in ABS/RC, as TR_0/RC measures the maximum trapping flux by active RCs (Yusuf et al., 2010). In our study, although TR_0/RC did increase, it did so to a lesser extent compared to ABS/RC. This suggests that the herbicide treatment induced both the inactivation of some RCs and an expansion of the functional antenna size.

The emergence of the positive K-band (Fig. 3F) also suggests this mechanism wherein plants increase the effective size of the antenna to compensate for the inactivation of some RCs and enhance ABS capacity. However, the inactivation of some oxygen-evolving complexes (OEC) (Fig. 3A and 3B) likely explains the inability of treated plants to fully photochemically process this energy. Additionally, the presence of the positive L-band (Fig. 3F) signifies diminished connectivity among photosystem II (PSII) units, which reduces their stability and results in decreased utilization of excitation energy in treated plants. Consequently, a significant proportion of the absorbed energy was dissipated non-photochemically (DI_0/RC) to prevent oxidative damage from excessive energy (Bassi and Dall'Osto, 2021).

Parameters of quantum yield and efficiency (Fig. 3A and 3B), which assess the effectiveness of absorbed energy in driving electron transport, revealed that the decline in electron transport chain (ETC) capacity primarily occurs after the reduction of QA. Despite an increase in TR_0/RC, the quantum yield of primary photochemistry (ϕ_{Po}) exhibited only a minimal reduction, indicating that the energy absorbed by antenna pigments and used for electron release from chl *a* in active RCs was relatively preserved. However, significant disturbances were observed in subsequent stages of the ETC. Notable decreases were recorded in the efficiency/probability of electron transfer beyond quinone A (QA^-) (ψ_{Eo}) and the quantum yield for electron transport in intersystem acceptors (ϕ_{Eo}). Additionally, reductions in parameters reflecting the final stages of the ETC were evident, including the efficiency/probability of electron transfer to reduce end electron acceptors at the PSI acceptor side (δ_{Ro}) and the quantum yield for this reduction (ϕ_{Ro}). Overall, performance indices incorporating energy fluxes and their bifurcations indicated that treated plants experienced a marked reduction in their capacity for energy conservation and efficient electron transport throughout the photosynthetic process. Both the potential for energy

conservation from exciton to intersystem electron acceptors (PI_{abs}) and to final electron acceptors in PSI (PI_{total}) exhibited significant declines following herbicide exposure.

The observed declines in photochemical efficiency directly impact the ability of treated plants to assimilate CO_2 (Baker and Oxborough 2004). The disruption in ETC compromises the overall capacity of the photosynthetic machinery, leading to decreased efficiency in the conversion of light energy into chemical energy. Our results indicate that the impact of IMI on ETC significantly affected the net assimilation rate (*A*) of *P. crassipes* even with high intracellular CO_2 concentration (C_i), likely due to reduced production of NADPH and ATP, given the strong impairment of electron flow to the final acceptors at the PSI acceptor side.

According to Scarponi et al. (1996), a decline in *A* can result from the inhibition of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) through a feedback mechanism triggered by high carbohydrate accumulation in the leaves. Indeed, treated plants exhibited elevated levels of total soluble sugars (TSS) and sucrose in their leaves. However, the relationship between the reduction in *A* and the decreased availability of ATP and NADPH is further supported by a concomitant decline in photorespiration, as evidenced by reduced glycolate oxidase (GO) activity. In conditions where *A* decreases but ATP and NADPH remain abundant, these molecules are typically diverted to photorespiration to maintain redox balance (ADP + NADP ⇆ ATP + NADPH) (Dusenge et al., 2019), a response that was notably diminished in this study. Consequently, the observed increase in C_i is likely due to stomatal closure and decreased stomatal conductance (g_s), leading to reduced CO_2 diffusion into leaf tissues. This elevated C_i appears to originate primarily from mitochondrial respiration, as the GO activity did not support a photorespiratory origin for the increased intracellular CO_2.

The oxidative stress analysis revealed distinct patterns between leaves and roots of treated plants, which can be interpreted in the context of the adverse effects induced by herbicide exposure. In the leaves, elevated levels of H_2O_2 were observed, along with clear signs of oxidative stress, such as increased lipid peroxidation (Fig. 6C). These symptoms are directly linked to disruptions in ETC during photosynthesis, which can increase the likelihood of electron leakage and the formation of reactive oxygen species (ROS) detrimental to leaf cells (Cobb 2021; Roach and Krieger-Liszkay 2019). However, despite the expectation that electron leakage from the ETC could lead to the formation of superoxide anions ($O_2^{\bullet -}$), the low levels of this primary ROS, combined with reduced superoxide dismutase (SOD) activity, suggest that oxidative stress in the leaves is not primarily due to this mechanism. Instead, the increased lipid peroxidation appears to be associated with elevated H_2O_2 levels.

Roots demonstrated they were also compromised by oxidative stress through increased electrolyte leakage at 14 DAA (Fig. 6D) but maintained some efficiency in the antioxidant defense system, as there was an increase in CAT and APX enzyme activity, while there was no significant difference in the levels of reactive oxygen species analyzed (H_2O_2 and $O_2^{\bullet -}$). Thus, the exact mechanisms underlying the oxidative stress observed in both leaves and roots of treated plants remain unclear. Zabalza et al. (2007) noted that in pea plants treated with imazethapyr, oxidative stress and changes in the antioxidant system appeared to be secondary and did not significantly contribute to the lethality associated with ALS inhibitors. Consequently, more detailed investigations are required to elucidate the precise nature of oxidative stress induced by IMIs in *P. crassipes* to better understand their effects.

Although the previous results demonstrated various adverse effects in treated plants, which would typically lead to the expectation of a reduction in ALS activity, the data from the analyzed mature leaves exhibited contrary behavior. The data revealed that ALS activity, along with amino acid and protein levels, was not

negatively impacted in leaves and roots of treated plants. In fact, ALS activity in the leaves was higher compared to the untreated group (Fig. 8A). In plant species and biotypes that exhibit herbicide tolerance, an initial suppression of ALS activity can be reversed through metabolic processes (Vidal and Winkler 2002). This reversal could explain cases where ALS activity remains unaffected post-herbicide exposure. However, it is possible that the herbicide did not reach the ALS in analyzed tissues of treated plants. This is because GST activity, an enzyme involved in the detoxification process of chemical contaminants (Strange et al. 2000; Severo et al. 2020), also did not show increased activity in these same analyzed tissues. Additionally, Ochogavía et al. (2014) studied the expression pattern of the ALS gene family and ALS activity in sunflower, observing that ALS transcript levels are highest in young leaves and reproductive tissues, and suggesting the existence of tissue-specific regulatory mechanisms at the transcriptional level. Thus, the observed increase in ALS activity in mature tissues during our experiment may represent a regulatory response to ALS inhibition in new tissues, which were severely affected, as evidenced in Experiment I where the growth of new tissues was significantly impaired in all doses. This could lead to an upregulation of ALS activity in mature tissues as a compensatory mechanism.

Conclusion

Although the herbicide did not negatively impact ALS activity in the mature leaf tissues analyzed in this study, other analyses, including visual symptoms, growth parameters, and physiological and biochemical assessments, indicate that the herbicide's mode of action still exerts significant secondary effects on these older tissues. Conversely, the disruption of new tissue development, even at the lowest dose, such as new leaves and plantlets (the primary reproductive mechanism of *P. crassipes*) highlights the herbicide's high toxicity to this species. This suggests that new tissues may be more directly susceptible to the herbicide's mode of action, and the observed increase in ALS activity in older tissues could represent a compensatory response to ALS suppression in new tissues. Consequently, further research is needed to explore this potential compensatory mechanism and better understand how the herbicide's mode of action differentially affects tissues at various developmental stages in *P. crassipes*.

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CONSIDERAÇÕES FINAIS

Os resultados deste estudo evidenciam o potencial risco dos herbicidas imidazolinonas para macrófitas que habitam ambientes aquáticos adjacentes aos cultivos de arroz no Rio Grande do Sul. Embora as análises dos tecidos maduros não tenham demonstrado que a atividade da ALS fora afetada pelo mecanismo de ação do herbicida, os parâmetros de crescimento, sintomas visuais, e outros parâmetros fisiológicos e bioquímicos indicaram que o herbicida teve capacidade significativa de desencadear efeitos tóxicos em plantas adultas de *P. crassipes* após 14 dias de tratamento.

O herbicida levou a clorose das lâminas foliares, desencadeando outros distúrbios nas plantas, como declínio na eficiência fotossintética, causando uma diminuição da conservação de energia ao longo da cadeia de transporte de elétrons, principalmente após a redução de Quinona A, o que acarretou no declínio da assimilação de CO_2 no metabolismo fotossintético do carbono. Levou também a outros distúrbios, como a diminuição na fotorrespiração, no comportamento estomático, acúmulo de carboidratos nas folhas e indício de estresse oxidativo, como peroxidação de lipídio nas folhas e extravasamento de eletrólitos nas raízes. Além disso, mesmo a menor dose utilizada na avaliação dos parâmetros de crescimento (0.2 mg L^{-1}) o herbicida foi capaz de interromper o desenvolvimento de broto, seu principal mecanismo reprodutivo.

As macrófitas aquáticas desempenham papéis essenciais nos ecossistemas aquáticos, como filtragem de poluentes, produtores primários, servem alimento e habitat para outros organismos, e estão diretamente relacionados com a produtividade e biodiversidade desses ambientes que habitam. Nesse sentido, a presença e a saúde dessas plantas são cruciais para garantir o desenvolvimento de suas funções vitais nesses ambientes, como a manutenção da qualidade da água e do equilíbrio ecológico. Assim, a contaminação dos corpos d'água por IMI derivados das áreas de cultivo de arroz pode ter efeitos adversos em cadeia, afetando a biodiversidade, a estrutura do habitat e a qualidade da água.

Ademais, estudos adicionais são necessários para avaliar de forma mais abrangente os efeitos dos herbicidas em diferentes estágios de desenvolvimento de *P. crassipes* e suas implicações para a saúde e funcionamento dos ambientes aquáticos. Seria interessante incluir análises de translocação do herbicida com rastreamento de isótopo do carbono e avaliar as interferências do mecanismo de ação, como por exemplo a atividade da ALS, índice específicos de BCAAs e proteínas em diferentes órgãos, o que permitiria uma compreensão mais abrangente dos seus efeitos em diferentes regiões da planta. Além disso, replicar as doses

utilizadas no Experimento I ao Experimento II permitiria estabelecer relações mais precisas entre a intensidade da toxicidade e a concentração aplicada, gerando dados mais robustos sobre a resposta das plantas ao tratamento.