UFRJ

CENTRO DE CIÊNCIAS DA SAÚDE

CURSO DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA VEGETAL E BIOPROCESSOS

TESE

***Setaria viridis* como modelo fisiológico de planta C_4 em estudos com herbicidas e déficit hídrico por estresse osmótico**

David da Cunha Valença

2020

UNIVERSIDADE FEDERAL DO RIO DE JANEIRO

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Setaria viridis COMO MODELO FISIOLÓGICO DE PLANTA C_4 EM ESTUDOS COM HERBICIDAS E DÉFICIT HÍDRICO POR ESTRESSE OSMÓTICO

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Leonardo Oliveira Medici

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Tese submetida como requisito parcial para obtenção do grau de **Doutor em Ciências (Biotecnologia Vegetal e Bioprocessos),** no curso de pós-graduação em Biotecnologia Vegetal e Bioprocessos.

Rio de Janeiro, RJ

Agosto de 2020

CIP - Catalogação na Publicação

d152s

da Cunha Valença, David
Setaria viridis como modelo fisiológico de planta C4 em estudos com herbicidas e déficit hídrico por estresse osmótico / David da Cunha Valença. -- Rio de Janeiro, 2020.
123 f.

Orientadora: Fernanda Reinert.
Coorientadora: Leonardo Medici.
Tese (doutorado) - Universidade Federal do Rio de Janeiro, Museu Nacional, Programa de Pós-Graduação em Ciências Biológicas (Botânica), 2020.

1. Setaria viridis. 2. Fisiologia vegetal. 3. modelo genômico. 4. Bioquímica. 5. qPCR. I. Reinert, Fernanda, orient. II. Medici, Leonardo, coorient. III. Título.

Elaborado pelo Sistema de Geração Automática da UFRJ com os dados fornecidos pelo(a) autor(a), sob a responsabilidade de Miguel Romeu Amorim Neto - CRB-7/6283.

Universidade Federal do Rio de Janeiro
Centro de Ciências da Saúde

Coordenação de Pós-Graduação em Biotecnologia Vegetal e Bioprocessos

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Aos quatro dias do mês de setembro do ano de dois mil e vinte, às 10 horas, reuniu-se via videoconferência, a Banca Examinadora abaixo discriminada, para avaliação da Tese de Doutorado do aluno **David da Cunha Valença**, intitulada: "*Setaria viridis* como modelo fisiológico de planta C_4 em estudos com herbicidas e déficit hídrico por estresse osmótico" desenvolvida sob a orientação da **Profª. Fernanda Reinert**, **Profª. Bianca Ortiz da Silva e Prof. Leonardo Oliveira Medici.** A apresentação feita pelo candidato foi acompanhada da arguição pelos componentes da Banca. Em seguida, esta se reuniu para sua avaliação e a tese foi (A) (inserir letra apropriada).

A) Aprovado;
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Rio de Janeiro, 04 de setembro de 2020.

Universidade Federal do Rio de Janeiro
Centro de Ciências da Saúde

Coordenação de Pós-Graduação em Biotecnologia Vegetal e Bioprocessos

ATA DO EXAME DE DEFESA DE TESE DE DOUTORADO DE DAVID DA CUNHA VALENÇA, COMO PARTE DOS REQUISITOS NECESSÁRIOS À OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS (BIOTECNOLOGIA VEGETAL E BIOPROCESSOS).

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DEDICATÓRIA

O que foi feito amigo
De tudo que a gente sonhou
O que foi feito da vida
O que foi feito do amor

Quisera encontrar
Aquele verso menino
Que escrevi há tantos anos atrás
Falo assim sem saudade
Falo assim por saber
Se muito vale o já feito
Mais vale o que será
E o que foi feito
É preciso conhecer
Para melhor prosseguir

Falo assim sem tristeza
Falo por acreditar
Que é cobrando o que fomos
Que nós iremos crescer
Outros outubros virão
Outras manhãs plenas de sol e de luz

Milton Nascimento

Aos meus pais Francisco Carlos Valença e Elizabeth da Cunha Souza Valença.

À minha avozinha amada Dulce Rosa Valença (in memoriam)

AGRADECIMENTOS

À Deus, pela vida e bênçãos concedidas.

Aos meus pais Francisco Carlos Valença e Elizabeth da Cunha Souza Valença, irmã Débora da Cunha Valença Oliveira e cunhado Marcus Oliveira, pelo amor a mim dedicado, pelo exemplo de família e orações.

À minha orientadora Professora Fernanda Reinert, pela amizade, paciência, ensinamentos e principalmente por sempre acreditar em mim e me oferecer apoio em todos os momentos.

Ao meus coorientadores e amigos Professores Leonardo Oliveira Medici e Bianca Ortiz da Silva por acreditarem em mim, pela amizade e apoio nas mais diversas situações.

Aos professores Ricardo Cardoso Vieira, Conan Ayade Salvador, Andrew Macrae, Marcio Alves Ferreira, Camila Ferreira de Pinho, Ricardo Chaloub, Cristina Moll Huther e Daniel Fonseca de Carvalho, pelos ensinamentos, apoio acadêmico e confiança.

Ao meu grande amigo, irmão de coração Ahmed Roland Zubiate Augustin, pela motivação, companheirismo e amizade.

À minha grande amiga Daniela Gomes, pelo carinho, companheirismo nos experimentos e ajudas em vários momentos.

À Universidade Federal do Rio de Janeiro pela oportunidade de acesso à educação de nível superior de qualidade, dada não só a mim, mas a muito brasileiros e estrangeiros.

Ao CNPq pela concessão da bolsa.

Aos amigos do Laboratório de Fisiologia Vegetal, Ana Carolina, Marcus Catten, Diego Lelis, Gabriel e Vanessinha, pelo apoio e amizade.

Aos amigos João Gabriel, Camilla Bottino, Bethânia Castro, João Paulo Silva, Bárbara Dias, Fernanda Costa, Letycia Ribeiro e Matheus Encarnação pela amizade, momentos de diversão e carinho.

Aos amigos das caronas ao fundão, que tornaram as idas e vinda muito mais agradáveis.

Ao grupo de pesquisa do laboratório de genética molecular vegetal (LGMV-UFRJ), principalmente aos colegas de trabalho e amigos: Stéfanie Moura, João Márcio, João Lins e Tamires Rodrigues e aos técnicos Luiz e Lina.

À Fazendinha Agropecuária do km 47, onde foi realizado os primeiros ensaios dessa tese, em especial aos funcionários Dione, Ivana, Helinho e Isaías.

Ao grupo de pesquisa Plantas Daninhas e Pesticidas no Ambiente (PDPA) da UFRRJ, com qual parceria foi realizado o primeiro trabalho desta tese (com herbicidas), em especial aos alunos que ajudaram diretamente nos experimentos.

À todos que de alguma forma me ajudaram até aqui.

RESUMO GERAL

VALENÇA, David da Cunha. ***Setaria viridis* como modelo fisiológico de planta C_4 em estudos com herbicidas e déficit hídrico por estresse osmótico.** 2020. 123p. Tese (Doutorado em Biotecnologia Vegetal e Bioprocessos). Instituto de Biologia, Departamento de Botânica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ.

O porte pequeno, o ciclo de vida curto e o genoma pequeno estão entre as características que sustentam no estabelecimento de *Setaria viridis* (L.) Beauv. como modelo de plantas com metabolismo fotossintético C_4. O uso de *S. viridis* como ferramenta biotecnológica tem mostrado grandes avanços em pesquisas em diversas áreas principalmente na última década. O objetivo desse trabalho foi avaliar a resposta dessa Poaceae como modelo de plantas C_4 sob dois tipos de estresse, aplicação de herbicidas e déficit hídrico. No primeiro experimento, foram avaliadas as alterações morfo-anatômicas e ultraestruturais de *S. viridis* em dois tempos (24 e 96 h) após a aplicação de clomazone e saflufenacil. Esses dois herbicidas são largamente utilizados e possuem mecanismos de ação que afetam, de formas diferentes, a fotossíntese, causando principalmente o aumento de processos oxidativos na planta. No segundo experimento, *S. viridis* foi submetida ao déficit hídrico por estresse osmótico utilizando uma concentração de 7,5% [g mL^{-1}] de polietilenoglicol 8000 (PEG-8000) durante 10 dias. Foram avaliadas características fisiológicas como fluorescência da clorofila *a*, condutância estomática (g_s), conteúdo de prolina, extravasamento de eletrólitos (EE), potencial osmótico e conteúdo relativo de água. Além disso, a análise qPCR foi usada para avaliar a expressão de cinco genes selecionados nas raízes por estarem implicados em resposta a seca (*SvLEA*, *SvDREB1C*, *SvPIP2-1*, *SvHSP20* e *SvP5CS2*). Em ambos experimentos, *S. viridis* apresentou resultados consistentes que reafirmam o uso dessa ferramenta como modelo de plantas C_4. Os dois herbicidas apresentaram efeitos visuais em *S. viridis* conforme descrito para o modo de ação de cada um. A anatomia e ultraestrutura mostraram danos drásticos e irreversíveis, como disrupção de membranas e deformações em organelas como os cloroplastos do mesofilo e da bainha vascular. No estudo de déficit hídrico, os parâmetros de fluorescência da clorofila *a* e a g_s foram drasticamente afetadas até o dia 6 após a imposição do estresse. No entanto, de modo contra-intuitivo, houve parcial recuperação de alguns parâmetros fotossintéticos (como F_V/F_M, ABS/RC, e DI_0/RC) e na g_s no 10º dia ainda sob déficit hídrico. O EE apresentou redução em plantas sob déficit hídrico, sugerindo um investimento em proteção à integridade de membranas pela expressão de osmoreguladores como deydrina (*SvLEA*) e prolina (*SvP5CS2*). Dessa forma, espera-se que os resultados apresentados aqui, com clomazone e saflufenacil, sirvam como baluarte no estabelecimento de *S. viridis* em estudos com herbicidas. Além disso, os nossos resultados destacando uma melhoria nas características fisiológicas de *S. viridis* (acesso A.10) sob déficit hídrico por estresse osmótico de longo prazo poderão ser úteis para melhor compreensão da resposta de Panicoideae C_4 a seca.

Palavras chave: clomazone; saflufenacil; prolina; fluorescência da clorofila *a*; análise qPCR.

GENERAL ABSTRACT

VALENÇA, David da Cunha. ***Setaria viridis* as a physiological model of C_4 plant in studies with herbicides and water deficit by osmotic stress.** 2020. 123p. Thesis (PhD in Plant Biotechnology and Bioprocesses). Institute of Biology, Department of Botany, Federal University of Rio de Janeiro, Rio de Janeiro, RJ.

The small size, short life cycle and small genome are among the resources that support the establishment of *Setaria viridis* (L.) Beauv. as a model of plants with C_4 photosynthetic metabolism. The use of *S. viridis* as a biotechnological tool has shown great advances in research in several areas, mainly in the last decade. The goal of this work was to evaluate the response of this Poaceae as C_4 plant model to two types of stress, herbicide application and water deficit. In the first experiment, they were evaluated as morpho-anatomical and ultrastructural changes of *S. viridis* in two times (24 and 96 h) after clomazone and saflufenacil application. These two herbicides are widely used and they have mechanisms of action that affect photosynthesis in different ways, mainly increasing oxidative processes in the plant. In the second experiment, *S. viridis* was subjected to water deficit by osmotic stress, using a 7.5% [g mL^{-1}] concentration of polyethylene glycol 8000 (PEG-8000) for 10 days. Physiological characteristics such as chlorophyll a fluorescence, stomatal conductance (g_s), proline content, electrolyte leakage (EL), osmotic potential and relative water content were evaluated. In addition, qPCR analysis was used to evaluate the expression of five selected genes in the roots as it is implicated in response to drought (*SvLEA*, *SvDREB1C*, *SvPIP2-1*, *SvHSP20* and *SvP5CS2*). In both experiments, *S. viridis* showed consistent results that reaffirm the use of this tool as the C_4 plant model. The two herbicides showed visual effects on *S. viridis*, as described for the mode of action of each one. Anatomy and ultrastructure showed drastic and irreversible damage, such as rupture of membranes and deformations in organelles, such as mesophyll and bundle sheaths chloroplasts. In the study of water deficit, the chlorophyll *a* fluorescence parameters and g_s were drastically affected until day 6 after stress imposition. However, in a counter-intuitive way, there was partial recovery of some photosynthetic parameters (such as F_V/F_M, ABS/RC and DI_0/RC) and g_s on the 10th day still under water deficit. The EL showed a reduction in plants under water deficit, suggesting an investment in protecting to integrity of membranes by the expression of osmoregulators such as dehydrin (*SvLEA*) and proline (*SvP5CS2*). Thus, it is expected that the results presented here, with clomazone and saflufenacil, serve as a bulwark in the establishment of *S. viridis* in studies with herbicides. In addition, our results highlight an improvement in the physiological characteristics of *S. viridis* (A.10 accession) under water deficit by long-term osmotic stress and may be useful to improve the response of Panicoideae C_4 to drought.

Key words: clomazone; saflufenacil; proline; chlorophyll *a* fluorescence; qPCR analysis.

ÍNDICE DE TABELAS

Tabela 1: Herbicidas e suas respectivas concentrações da dose comercial aplicada sobre S*etaria viridis*. 29

ÍNDICE DE FIGURAS

Figura 1. Planta de *Setaria viridis*. Fonte: Acervo do grupo de pesquisa do laboratório de Fisiologia vegetal/ UFRJ. 14

Figura 2: Seção transversal da folha de *Setaria viridis.* (A) Micrografia ótica evidenciando a anatomia *Kranz.* (B) Micrografia eletrônica apresentando a disposição dos cloroplastos dentro das células da bainha e do mesofilo. (*) Células da bainha vascular ao redor dos feixes vasculares; (#) células do mesofilo entre os feixes vasculares; BV: bainha vascular; M: Mesofilo; e CL: cloroplasto. Barras de escala: 100 (A) e 10 (B) µm. Fonte: Acervo do grupo de pesquisa do laboratório de Fisiologia vegetal/ UFRJ. 16

Figura 3: Câmara de crescimento, tipo Fitotron, localizada no Laboratório de Genética Molecular Vegetal (LGMV-UFRJ) com diferentes experimentos. 27

Figura 4: Aplicação do herbicida (A) e vasos com plantas dispostas na casa-de-vegetação após a aplicação (B). 30

Figura 5: Montagem do “berçário” para germinação das sementes de *Setaria viridis*. Componentes: 1. Gargalos de garrafa PET; 2. Tulle; e 3. Elástico. Barras de escala: 2 cm.34

Figura 6: (A-D) Montagem do sistema hidropônico individual. Componentes: 1. Copo plástico preto (200 mL) envolvido por papel alumínio; 2. Copo plástico transparente (200 mL); e 3. Tampa plástica preta envolvida por papel alumínio cortada no centro para inserção de gargalo de PET. (E-F) Sistema de hidroponia montado com as plantas apoiadas acima das tampas com as raízes em solução (imagens sem o componente 1). Barras de escala: 2 cm. 35

SUMÁRIO

RESUMO GERAL **VII**

GENERAL ABSTRACT **VIII**

1. INTRODUÇÃO **13**

1.1. O gênero *Setaria* 13

1.2. O uso de *Setaria viridis* como modelo C_4 biotecnológico 14

1.3. Herbicidas e o uso de *S. viridis* em testes com clomazone e saflufenacil 17

1.4. Restrição hídrica e o modelo C_4 de *Setaria viridis* 19

2. OBJETIVO **19**

2.1. Objetivos específicos 26

3. MATERIAL E MÉTODOS **27**

3.1. Crescimento das plantas 27

3.2. Aplicação dos herbicidas e desenho experimental 28

3.3. Avaliação de injurias foliares 30

3.4. Determinação do conteúdo de clorofilas *a* e *b* e carotenoides 30

3.5. Análises anatômicas, micromorfológicas e ultraestruturais 31

3.6. Análises estatísticas dos dados 32

3.7. Crescimento das plantas 33

3.8. Tratamentos e desenho experimental 36

3.9. Fluorescência da clorofila *a* 36

3.10. Análises biométricas e condutância estomática 37

3.11. Conteúdo relativo de água (CRA) 38

3.12. Extravasamento de eletrólitos (*leakage*) 38

3.13. Medição do potencial osmótico 38

3.14. Conteúdo de prolina 39

3.15. Análise de dados 39

3.16. Análise de expressão gênica 39

4. CAPÍTULO I – ARTIGO I 41

Changes in Leaf Blade Morphology and Anatomy Caused by Clomazone and Saflufenacil Observed in *Setaria viridis* a Model C_4 Plant 41

5. CAPÍTULO II – ARTIGO II 70

Physiological and molecular responses of *Setaria viridis* to osmotic stress 70

6. CONSIDERAÇÕES FINAIS E PERSPECTIVAS 108

7. REFERÊNCIAS BIBLIOGRÁFICAS 110

ANEXOS 119

1. INTRODUÇÃO

1.1. O gênero *Setaria*

As monocotiledôneas, as quais pertencem a um subgrupo das angiospermas, têm aproximadamente 52 mil espécies, e representa 22% deste subgrupo. Neste, se destacam algumas plantas monocotiledôneas de interesse para alimentação humana e/ou animal, ou para a produção de biocombustíveis; tais como as gramas, o milho, o arroz, a cana-de-açúcar, o trigo, o centeio, entre outras (BORGES *et al.*, 2017; FRANKE *et al.*, 2017; WALTER *et al.*, 2017). As espécies monocotiledôneas desse subgrupo das angiospermas possuem raízes do tipo fasciculadas, com origem em um único ponto, e folhas paralelinérveas. Além disso, possuem apenas um cotilédone na semente e as substâncias que nutrem o grão ficam armazenadas no endosperma. A família Poaceae constitui 17% das monocotiledôneas, sendo caracterizada por ter folhas alternas, dísticas e formadas por bainha, lâmina e língula (PANT *et al.*, 2016).

O gênero *Setaria* é membro da subtribo Cenchrinae, tribo Paniceae, subfamília Panicoideae, família Poaceae, ordem Poales e grupo das monocotiledôneas (PANT *et al.*, 2016). Neste gênero, inclui quase 100 espécies de gramíneas panicóides. Os indivíduos pertencentes a esta subtribo Cenchrinae tem como características a presença de ramos estéreis na inflorescência. As gramíneas pertencentes à subfamília Panicoideae estão entre as de maior importância agronômica, tais como o milho, o sorgo e a cana-de-açúcar (LI & BRUTNEL, 2011).

A *Setaria viridis* (L.) P. Beauv. (Figura 1) é uma planta herbácea anual que possui altura entre 10 a 15 cm, seu ciclo de vida é de aproximadamente oito semanas, com produção de cerca de 13 mil sementes por planta (LI & BRUTNEL, 2011). A planta desta espécie possui inflorescência bissexual e sementes de genoma diploide (2n=18). O cultivo da *Setaria viridis* começou há cerca de 5.900 a.c., no noroeste da China (BARTON *et al.*, 2009), e esta espécie é nativa de partes da África, Ásia temperada, Ásia tropical e Europa (DEKKER, 2003).

Figura 1: Planta de *Setaria viridis*. Fonte: Acervo do grupo de pesquisa do laboratório de Fisiologia vegetal/ UFRJ.

S. viridis possui o metabolismo do tipo C_4, do subtipo NADP-ME, que é a mesma das plantas C_4 de maior interesse agronômico, como o milho e a cana-de-açúcar. As trocas gasosas ocorrem em ambas faces foliares (ada e abaxial), pois a folha é do tipo anfiestomática, com maior densidade de estômatos na superfície adaxial (SANYAL *et al*., 2006). A *S. viridis* pode ser um modelo interessante de planta a ser estudado, para tentar entender os efeitos dos fatores abióticos sobre o processo fisiológico das culturas de interesse agronômico (notadamente as do metabolismo C_4). O fator abiótico é um dos desafios agronômicos, os quais permeiam a agricultura mundial (LI & BRUTNEL, 2011).

1.2. O uso de *Setaria viridis* como modelo C_4 biotecnológico

A utilização de organismos como modelos experimentais permite que estudos laboratoriais possam ser realizados de forma mais rápida e facilitada (SIBOUT, 2017). Isto é possível sempre que esses modelos apresentam características que facilitam a investigação, tais como: pequeno porte da planta, ciclo curto de vida e modificação genética viável do material a ser estudado (AMARAL, 2014).

Diversos tipos de estudos são realizados com a espécie *Arabidopsis thaliana* (RABANAL *et al*., 2017; SELVARAJ *et al*., 2017; SHAH *et al*., 2017). Esta espécie é uma herbácea da família Brassicaceae, e esta planta foi a primeira a ter o genoma completamente

sequenciado (BRITO *et al*., 2009). Inúmeros desses estudos desvendaram uma série de características fisiológicas e de rotas metabólicas comuns a diversas famílias botânicas (LEONELLI, 2017).

Por outro lado, quando se vislumbra o modelo C_4 de *S. viridis*, pouco se sabe sobre os processos de desenvolvimento e vias metabólicas envolvidos (BRUTNELL *et* al., 2010; SIBOUT, 2017). No entanto, significativo esforço tem sido conferido às pesquisas relacionadas ao desenvolvimento de ferramentas para se estudar a *S. viridis* (BRUTNELL *et al*., 2010; LI & BRUTNEL, 2011).

Dentre as características associadas a essa espécie, e que motivam a utilização como planta modelo, está a assimilação de CO_2 ser por meio do metabolismo fotossintético do tipo C_4 (BRUTNELL *et al*., 2010). Nesse sistema de assimilação de CO_2, o gás que entra nas células do mesofilo por meio dos estômatos é convertido a bicarbonato, fixado em oxalacetato e então reduzido a malato no cloroplasto do mesofilo (no tipo NADP-ME) ou a aspartato no citosol (nos tipos NAD-ME e PECK), de onde se difunde para as células da bainha do feixe vascular (MENDONÇA, 2017). Essa compartimentação dos processos também é possível graças a anatomia do tipo *Kranz* presente na grande maioria das plantas C_4, e também em algumas C_3 (SAGE; KHOSHRAVESH; SAGE, 2014).

No gênero *Setaria*, a anatomia *Kranz* possui, além da camada mais externa próxima à epiderme denominada mesofilo, uma camada interna circunvizinha ao feixe vascular, chamada de bainha vascular (EDWARDS & VOZNESENSKAYA, 2011; PAK *et al*., 1997) (Figura 2). Ao chegar ao cloroplasto da bainha, o malato pode ser descarboxilado, dependendo da espécie, pelas enzimas NADP-ME, NAD-ME ou PEPCK, e somente assim ocorre a liberação do CO_2, próximo aos sítios de carboxilação da ribulose 1,5-bifosfato carboxilase/oxigenasse (Rubisco) (FURBANK, 2011). Este processo permite que um ambiente rico em CO_2 seja produzido em torno da enzima Rubisco o que evita as perdas associadas à fotorrespiração (BRUTNELL *et al.*, 2010).

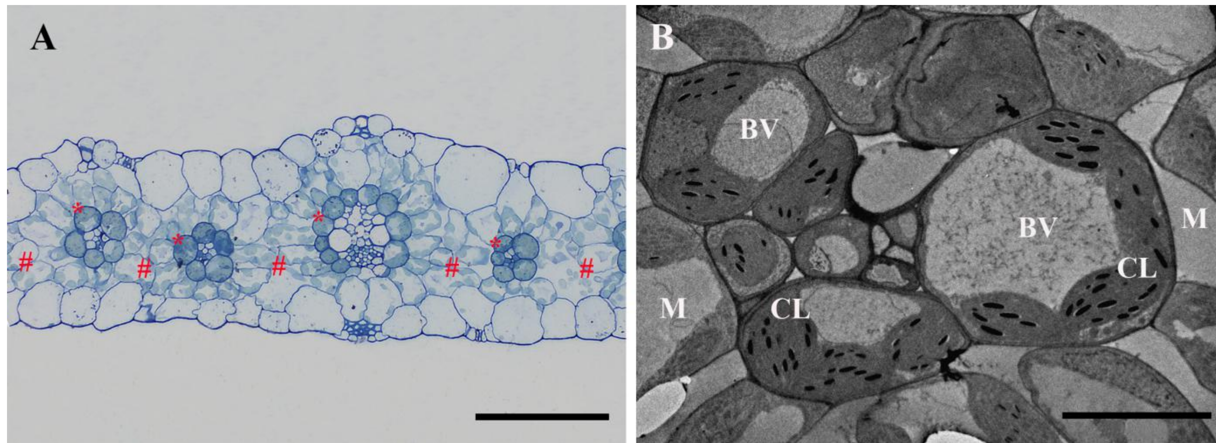

Figura 2: Seção transversal da folha de *Setaria viridis.* (A) Micrografia ótica evidenciando a anatomia *Kranz.* (B) Micrografia eletrônica apresentando a disposição dos cloroplastos dentro das células da bainha e do mesofilo. (*) Células da bainha vascular ao redor dos feixes vasculares; (#) células do mesofilo entre os feixes vasculares; BV: bainha vascular; M: Mesofilo; e CL: cloroplasto. Barras de escala: 100 (A) e 10 (B) µm. Fonte: Acervo do grupo de pesquisa do laboratório de Fisiologia vegetal/ UFRJ.

A maior atividade carboxilase da Rubisco nas células da bainha incrementa a eficiência intrínseca no uso da água (iEUA=A/gs: sendo A: assimilação fotossintética do carbono e g_s: condutância estomática) e no nitrogênio (EUN=A/N) (EDWARDS *et al.*, 2010; EDWARDS & SMITH, 2010), pois, aumenta o rendimento fotossintético dessas classes de plantas. A manutenção da iEUA em valores elevados está relacionada à redução da condutância estomática, à medida que a fonte de CO_2 dessas espécies passa a ser os ácidos de quatro carbonos presentes nas células do mesofilo (SAGE, 2004). Em plantas C_4, a maior eficiência do uso do nitrogênio, quando comparadas às C_3, está relacionada ao menor investimento na concentração da Rubisco (KEELEY & RUNDEL, 2003). Isso pode explicar a maior distribuição global atual dessas espécies C_4 em regiões tropicais, subtropicais e temperadas quentes (SAGE, 2004), o que está associada a maior capacidade de uso do N com a maior assimilação fotossintética do carbono nessas regiões (SAGE *et al.*, 2012).

Por fim, o modelo de fotossíntese C_4 pode auxiliar em estudos experimentais de diversas espécies de importância alimentar e energética já consolidadas. Dentre essas espécies de utilização comercial estão o milho (*Zea mays*), o sorgo (*Sorghum bicolor*), a cana-de-açúcar (*Saccharum officinarum*), o milheto (*Panicum miliaceum*, *Pennisetum Glaucum* e *Setaria italica*), *Miscanthus* x *giganteus* e switchgrass (*Panicum virgatum*) (BRUTNELL *et al.*, 2010).

1.3. Herbicidas e o uso de *S. viridis* em testes com clomazone e saflufenacil

Por mais de um século, os herbicidas inorgânicos são utilizados no controle de plantas daninhas ao redor do mundo (KRAEHMER *et al*., 2014). Ainda assim, estima-se que cerca de 34% da produtividade na agricultura mundial é perdida por infestação de plantas invasoras (LI *et al*., 2017). Essas perdas muitas vezes ocorrem devido à resistência que é estabelecida após longo período de uso destes produtos. Por isso, o uso de pesticidas capazes de controlar o crescimento de plantas daninhas é de grande importância para a agricultura de alta eficiência produtiva (kg ha^{-1}) (DU *et al*., 2018). Mundialmente, muito se investe no estudo e desenvolvimento de novas fórmulas que sejam eficientes no controle das principais plantas consideradas daninhas (KRAEHMER *et al*., 2014), especialmente em casos em que haja resistência pré-estabelecida. Entretanto, ainda pouco se conhece sobre os efeitos desses herbicidas nas plantas. Quando se considera as espécies resistentes a diferentes tipos de herbicidas, calculam-se que 32% pertencem a família Poaceae (HEAP, 2019).

No Brasil, o produto Gamit®, que possui o ingrediente ativo denominado clomazone, é um herbicida importante, e utilizado em lavouras de algodão, arroz, cana-de-açúcar, mandioca e soja (BRASIL, 2020). O clomazone, [2 - [(2 - chlorophenyl) methyl] - 4,4 - dimethyl - 1,2 - oxazolidin - 3 - one], é um herbicida pertencente ao grupo químico das Isoxazolidinonas com mecanismo de ação na inibição da biossíntese de carotenoides (DE OLIVEIRA JR, 2011). Dentre as espécies que possuem seletividade a essa substância encontram-se duas Poaceas, o arroz e a cana-de-açúcar. O sintoma mais comum desse herbicida quando aplicado em plantas suscetíveis é a fotodregradação da clorofila, que ocorre logo devido ao bloqueio da síntese de carotenoides, o que causa como sintomatologia final a despigmentação das folhas (DE OLIVEIRA JR, 2011). Atualmente, o que se sabe sobre o clomazone é que ele se torna ativo como herbicida ao ser metabolizado a 5-ceto-clomazone pelas hemoproteínas do sistema citrocromo P-450 monoxigenase. A presença dessa substância então inibe a 1-deoxi-xilulose 5-fosfatase sintase (DOXP), que é um composto-chave na síntese de isoprenóides dos plastídeos (DE OLIVEIRA JR, 2011).

A característica como a alta solubilidade em água, confere ao clomazone um elevado potencial de persistência no ambiente, o que aumenta o risco de contaminação, além do surgimento de novas espécies tolerantes (CABRAL *et al*., 2017). Mesmo com os sintomas morfológicos característicos, o monitoramento da presença de herbicidas no ambiente ainda é limitado. Quando da utilização de plantas como bioindicadores, por exemplo, existe uma certa limitação pelo fato de as plantas, que são organismos extremamente sensíveis a diferentes

ações externas, poderem reagir de forma semelhante a diferentes poluentes (DE TEMMERMAN *et al*., 2004). Neste caso, as lesões visíveis como a clorose e a necrose, as quais são sintomas comuns à ação de herbicidas, poderiam ser causadas também por outros fatores ambientais, o que levaria a uma análise errônea do poluente analisado (CABRAL *et al*., 2017). Nesse contexto, análises de micromorfologia foliar destacam-se como ferramenta promissora na detecção de alterações estruturais como resposta a diferentes tipos de poluentes. (SANT'ANNA-SANTOS & AZEVEDO, 2006).

No Brasil, um outro herbicida largamente utilizado em culturas como o arroz, a mandioca, a cana-de-açúcar, o milho e as pastagens é o produto comercial denominado Heat®, que possui o ingrediente ativo denominado saflufenacil (BRASIL, 2020). O saflufenacil, N' - [2 - chloro - 4 - fluoro - 5 - (1,2,3,6 - tetrahydro - 3 - methyl - 2,6 - dioxo - 4 - (trifluoromethyl) pyrimidin - 1 - yl) benzoyl] - N - isopropyl - N - methylsulfamide, é um herbicida pertencente ao grupo químico das birimidinadionas cujo mecanismo de ação inibe a atividade da enzima protoporfirinogênio oxidase (PPO), que está presente na membrana do cloroplasto (DE OLIVEIRA JR, 2011). A inibição dessa enzima que é responsável pela oxidação do protoporfirogênio à protoporfirina IX, ambos precursores da clorofila, causa o acúmulo do protoporfirogênio que acaba sendo difundido para fora do centro de reação (DE OLIVEIRA JR, 2011).

No citosol, ocorre a auto-oxidação do protoporfirinogênio por uma enzima insensível ao inibidor, formando uma protoporfirina IX diferente da que seria produzida pela via da enzima PPO (LANGARO *et al*., 2017). A protoporfirina IX gerada, reage então com o oxigênio molecular e a produção desta protoporfirina é estimulada pela ação da luz. Após, ocorre a formação do oxigênio singlete, que causa a peroxidação lipídica nas membranas celulares. Consequente, por ação desta molécula reativa de oxigênio, ocorre a degradação das clorofilas e dos carotenoides, bem como o rompimento de membranas (TRIPATHY; MOHAPATRA; GUPTA, 2007). Quando em contato com as folhas e em plantas suscetíveis, o saflufenacil possui pouca seletividade, e é observada necrose nas folhas poucas horas ou dias após a aplicação desta molécula.

O tamanho reduzido do genoma (aprox. 510 Mb), o pequeno porte da planta e o ciclo de vida de *S. viridis* (BRUTNELL *et al*., 2010) indicam que esta espécie pode ser adequada para avaliar os efeitos da ação dos herbicidas sobre o metabolismo da *S. viridis*. Em testes com herbicidas, o uso de técnicas para avaliar a estrutura e a ultraestrutura de lâminas foliares pode acelerar e dar mais confiabilidade ao modelo C_4 de *S. viridis*. Os efeitos do clomazone e

saflufenacil caso sejam claramente mostrados e descritos servirão de referência para aperfeiçoar o conhecimento relacionados à interação plantas C_4 e herbicidas. Semelhante ao modelo de planta mais famoso, que é o modelo associado à *Arabidopsis thaliana*, e que por meio de pesquisas que utilizam esta espécie, melhoraram e acelerraram os estudos em biologia vegetal, o conhecimento adquirido com o estudo desta planta modelo C4 *S. viridis* pode ter impactos positivos indiretos na segurança alimentar global e na compreensão dos mecanismos de ação de herbicidas.

1.4. Restrição hídrica e o modelo C_4 de *Setaria viridis*

As alterações climáticas impactam diversos setores da atividade humana, em particular a produção agrícola (ARAÚJO *et al*., 2016), seja por escassez ou por excesso de água (MARENGO, 2006). Segundo previsões climáticas, e entre 2030 e 2052, é esperado que o aquecimento global possa elevar a temperatura em 1,5 ºC (IPCC, 2019). Os modelos de alterações climáticas sugerem que possam ocorrer com maior frequência os eventos de seca severa e os de calor (FISCHER; KNUTTI, 2015; TESKEY *et al*., 2015). As consequências desse aumento de temperatura já são evidentes, como o derretimento das calotas polares, os recordes de calor em muitos locais e a seca em diversas regiões (FEARNSIDE, 2006).

Com áreas agrícolas afetadas por longos períodos de seca, e ao contínuo crescimento desses eventos, existe a preocupação dos impactos negativos no setor agrícola. Estes impactos podem reduzir a produtividade, visto a limitação hídrica deverá ser um fator decisivo na produção das culturas de interesse agronômico. Similarmente, frente às projeções de crescimento populacional até o ano de 2050, a produção de alimentos necessita ser incrementada em 70% em relação ao que é produzido atualmente (FAO, 2017). Dessa forma, os estudos relacionados à elucidação e análise dos mecanismos de aclimatação e de tolerância das plantas nestes ambientes com moderada e severa limitação hídrica são altamente necessários.

Em contraposição aos efeitos do aumento da temperatura do planeta nas regiões tropicais, em algumas regiões na latitude norte, como exemplo as regiões produtores de trigo do Canadá, estas poderão ter efeitos favoráveis deste fator ambiental sobre o aumento da produtividade agrícola (SMITH *et al.,* 2013). No cenário futuro, mesmo se considerados os efeitos acentuados da redução da disponibilidade hídrica no solo e no ar sobre o crescimento das culturas, pode-se ter o aumento da eficiência no uso da água, uma vez que é esperado um incremento de CO_2 ($\cong$ 800 a 1000 ppm em 2100) (SMITH *et al.,* 2013; TOZZI, 2013).

Algumas pesquisas mostram que o incremento de CO_2 pode mitigar o efeito da elevada temperatura e limitação hídrica imposta pelas mudanças climáticas globais (Rodrigues et al 2016; Dubberstein et al, 2020). Em função da alteração climática mundial, é esperada uma tendência de alteração no zoneamento agrícola do planeta Terra.

No cenário futuro de elevação nos eventos de seca, torna-se fundamental conhecer as respostas das plantas à limitação hídrica do solo e do ar, uma vez que a produção agrícola é limitada diretamente pela frequência e intensidade do déficit hídrico ao qual a cultura pode estar submetida (SANTOS & CARLESSO, 1998). Essa limitação de água pode ser responsável por cerca de 65% de variação da produção final, não considerando efeitos extremos (ORTOLANI & CAMARGO, 1987). No Brasil, as primeiras pesquisas mais elaboradas sobre esse tema constam da década de 90 (ALVES & EVENSON, 1996, 1998; LIMA *et al.,* 2001). Esses estudos indicaram que dentre as ações que devem ser exploradas como estratégia de adaptação às mudanças climáticas estão a busca de genótipos mais tolerantes a temperaturas elevadas, genótipos tolerantes à seca, o déficit de irrigação controlado (*regulated deficit irrigation*), ou a seca parcial do sistema radicular (*partial root-zone drying*).

A variação na temperatura e os efeitos desta variação sob todo o clima do planeta pode significar, por exemplo, a redução drástica na produção de milho e forte redução de áreas aptas para plantio de café em algumas regiões do Brasil (ASSAD *et al.,* 2004; LIMA & RODRIGUES*,* 2010). De maneira análoga, as mudanças nas precipitações, as quais afetam a produtividade vegetal, impactam negativamente os produtores agrícolas, assim como os valores da terra apta à agricultura (MOTTA *et al*., 2011). Assim, em vista dessa problemática, espera-se o desenvolvimento de ações de pesquisa de melhoramento genético, em associação com outras áreas importantes da botânica, como a fisiologia vegetal, a biologia molecular, e a bioquímica vegetal, objetivando a obtenção de variedades de plantas mais resistentes a temperaturas supra-ótima, juntamente com recursos tecnológicos associados ao uso eficiente da água na agricultura.

Por possuir uma relação evolutiva com culturas de importância agronômica/econômica, como sorgo e a cana-de-açúcar, o estudo da resposta de *S. viridis* associada a condições de estresse hídrico pode fornecer alternativas para a expansão e manutenção da agricultura em áreas marginais, onde a agricultura torna-se limitada, reduzindo assim o abandono de áreas agrícolas, além de produzir conhecimento da fisiologia vegetal básica para as futuras pesquisas. Essa gramínea *S. viridis,* a qual foi proposta à comunidade científica como sistema

genético modelo para os estudos sobre o metabolismo fotossintético C_4 (BRUTNELL *et al*., 2010), é uma erva daninha que convive bem em situações diversas ao redor do mundo (AUSTIN, 2006). A variedade de ambientes nos quais a *Setaria* sp. é encontrada, e a compatibilidade de cruzamento de *S. viridis*, sugere que o germoplasma dessa gramínea pode também ser uma fonte rica de variação genética com novas oportunidades para estudar os genes que controlam a tolerância à seca. Muitas informações básicas sobre recursos genéticos e moleculares não estão ainda explicadas e por isso não estão disponíveis em banco de dados (AMARAL, 2014).

De forma análoga, ocorre com características associadas à fisiologia da planta, o que de certa maneira poderia prejudicar o estabelecimento do modelo C_4 de *S. viridis.* Contudo, muito esforço tem sido realizado na última década, relacionado ao avanço de pesquisas com essa gramínea. Entre os anos 2010 e 2019, em comparação ao intervalo entre os anos de 2000 a 2009, o número de artigos publicados com *S. viridis* foi maior que o dobro (aproximadamente 215% maior). Desta maneira, a espécie *S. viridis*, sob condições limitadas de água no solo, já demonstrou produzir elevados níveis de biomassa, e mantendo a mesma eficiência de uso da água sob condições de capacidade de campo (FAHLGREN *et al*., 2015). O estudo de culturas capazes de apresentar um uso eficiente de água elevado pode ser de grande importância para a agricultura, num cenário futuro de limitações hídricas.

O déficit hídrico pode induzir a diminuição do potencial de água na célula, bem como causar o fechamento estomático e a produção de espécies reativas de oxigênio - ERO. Em nível celular, as membranas e as proteínas podem ser danificadas pela redução da hidratação e pelo aumento de ERO's. Ainda, a assimilação fotossintética do carbono pode ser reduzida pela diminuição da concentração de CO_2 nos cloroplastos, como resultado do fechamento estomático. Os recursos de defesa de cada cultura contra a desidratação e a eliminação de ERO's tem relação intrínseca com a tolerância à seca. A resposta das culturas à disponibilidade de água no solo vem da estreita relação dos processos fisiológicos, que estão diretamente ligados a produção vegetal, e outros fatores associados ao solo, à planta e a atmosfera (CARVALHO *et al*., 2012). Estudos laboratoriais mostraram que o polietilenoglicol (PEG) estabelece continuamente e mantém o forte potencial osmótico das soluções, servindo bem ao propósito de manipular a disponibilidade de água para plantas em qualquer configuração experimental (NIO *et al*., 2019).

O acesso A10.1 de *S. viridis* é um dos acessos mais resistente a reduzidos potenciais hídrico de solo (SAHA *et al*., 2016). Uma outra característica é a capacidade de recuperação

pós-irrigação, mesmo após um longo período de limitação hídrica do solo, principalmente a recuperação associada à taxa fotossintética líquida (LUO *et al*., 2009; LUO *et al*., 2011) e por manter uma correlação elevada e positiva entre a eficiência no uso da água, e o tamanho da planta (FELDMAN *et al*., 2018). A capacidade de tolerar a escassez de água no solo pode ter relação com a forma com que a espécie mantém a elevada eficiência no uso deste recurso, isto é, incrementando a assimilação de CO_2 por unidade de água transpirada (FELDMAN *et al*., 2018).

As plantas respondem às condições impostas pelo estresse hídrico de forma diferente, de acordo com a espécie, a cultivar e o tempo de exposição, entre outros fatores (GALLÉ *et al*., 2015). Como não há uma única variável fisiológica que indique, por si só, a tolerância da planta à seca, é importante avaliar diversas variáveis (FAHLGREN *et al*., 2015). As análises de potencial hídrico, condutância estomática, temperatura e transpiração foliar são consideradas importantes para avaliação das respostas das espécies vegetais ao estresse hídrico (LAWLOR, 2013). O conteúdo relativo em água (CRA) apesar de simples, pode apresentar-se como indicador da intensidade do estresse por seca, principalmente em plantas que não realizam ajuste osmótico (HSIAO, 1990), já que neste caso não há a acumulação de solutos, uma vez que o potencial osmótico é inversamente relacionado ao volume osmótico, que por sua vez, é frequentemente relacionado ao CRA (MORGAN, 1984).

A área foliar e a massa seca da parte aérea são variáveis sensíveis ao estresse hídrico (BLUM, 1997). A medição da emissão da fluorescência da clorofila *a* constitui uma das principais ferramentas utilizadas no estudo do porcesso fotossíntético (PAPAGEORGIO & GONVINDJEE, 2004). O método de monitoramento da indução de fluorescência é largamente utilizado para medir a atividade do fotossistema II, já que é não invasivo, fácil, rápido, e preciso, e requer equipamentos relativamente baratos (LAZÁR, 1999).

Um dos conceitos de eficiência no uso da água (EUA), é a relação entre biomassa produzida pela água aplicada, e pode ser um importante recurso a ser utilizado para avaliar a eficiência da cultura em condição de irrigação (MEDICI *et al*., 2014). A redução na aplicação de água de forma adequada, por meio de técnicas, como o déficit de irrigação controlado, ou a seca parcial do sistema radicular, pode aumentar a eficiência no uso da água. De fato, alguns estudos mostram uma maior produção sob disponibilidade hídrica adequada, sem desperdiçar água (BOYLE *et al*., 2015; MEDICI *et al*., 2014). Esses estudos demonstram, que a maior EUA pode ser alcançada com uma adequada diminuição da disponibilidade da água, não sendo necessário que a redução seja grande.

As plantas estabelecidas em solos com a umidade reduzida têm o potencial de água na folha mais negativo, para que possam estabelecer um gradiente que favoreça a absorção de água pelas raízes (NASCIMENTO *et al*., 2011). O estado de turgor das células é importante, pois pode afetar a fotossíntese e o crescimento celular, e tem a faixa ideal entre valores de 0 e 0,8 MPa (PETRY, 1991). Nesse sentido, as plantas são diretamente influenciadas pelas condições hídricas do solo, estabelecendo assim um adequado movimento de água no sistema solo-planta-atmosfera (MARINHO *et al*., 2016). Por exemplo, as plantas sob estresse hídrico moderado podem não demonstrar redução no rendimento quantico máxima do PSII, e a reduzida desidratação pode inibir apenas a expansão celular, o crescimento da planta e a condutância estomática (ARAÚJO & DEMINICIS, 2009). No entanto, sob estresse hídrico severo as plantas comumente estão submetidas a um efeito fotoinibitório, evidenciado por um decréscimo significativo do rendimento quântico máximo do PSII (F_V/F_M) (ARAÚJO & DEMINICIS, 2009). Dessa forma, o conhecimento da intensidade da desidratação na célula vegetal é um dos fatores importantes para a determinação da resposta da fotossíntese ao déficit hídrico.

A condutância estomática pode ser entendida como a taxa de passagem de vapor de água através dos estômatos das folhas, em relação ao máximo de vapor de água que tem na folha, numa dada temperatura foliar, e é frequentemente utilizada como índice do estado hídrico (CARDORIN *et al*., 2016). A abertura e/ou fechamento dos estômatos estão principalmente relacionados à intensidade/qualidade de luz e a hidratação das folhas. Desta forma, em estudos de restrição hídrica, a abertura/fechamento dos estômatos e a área foliar irão influenciar a produtividade do vegetal, visto que a abertura controla a absorção de CO_2 e área foliar da planta em associação com uma adequada arquitetura foliar determina a eficiência na interceptação de luz (COSTA *et al*., 2007). O fechamento estomático é uma das primeiras vias nas respostas das plantas ao déficit hídrico, o que evita a perda de água pela transpiração foliar (PELOSO *et al*., 2017). Esse fechamento pode estar associado tanto à sinalização química da raiz para a parte aérea, por meio da variação dos teores de ácido abscísico, pH e outros componentes químicos da seiva do xilema (WILKINSON & DAVIES, 2002), à desidratação das folhas (CHRISTMANN et al., 2007) ou ainda por sinais elétricos (GALLÉ *et al*., 2015) e hidráulicos (LIMPUS, 2009). De toda forma, qualquer que seja o primeiro sinal a induzir o fechamento estomático, esse acontecimento trará grandes consequências à fisiologia das plantas sob déficit hídrico.

Para a fotossíntese, com o fechamento estomático, há a limitação da difusão de CO_2 até os sítios de carboxilação da Rubisco, o que pode comprometer a fase bioquímica e a fase fotoquímica. Em plantas C_3, isso reduz drasticamente a assimilação de carbono (CHAVES & OLIVEIRA, 2004), porém em plantas C_4, apesar da restrição estomática reduzir de maneira considerável a fotossíntese, essa limitação é minimizada pelo mecanismo de concentração de CO_2, típico desse metabolismo C_4 (MARCOS, 2017).

Dessa forma, o déficit hídrico afeta de maneira diferente gramíneas com metabolismo do tipo C_3 e C_4. Em arroz, por exemplo, uma gramínea do tipo C_3, a taxa fotossintética diminuiu drasticamente durante a seca do solo, principalmente devido à diminuição da condutância estomática (WANG *et al*., 2018). Contudo, sob déficit hídrico, em cana-de-açúcar, uma gramínea do tipo C_4, foi relatado uma limitação bioquímica, principalmente relacionada à diminuição da velocidade de carboxilação da fosfoenolpiruvato carboxilase (PEPCase) e da Rubisco (CARMO-SILVA *et al*. 2008; MACHADO *et al*., 2013). Da mesma forma, para essa cultura, o déficit hídrico também causou a diminuição da eficiência operacional do PSII e na taxa de transporte de elétrons (SALES *et al*., 2013; SALES *et al*., 2015).

O déficit hídrico pode ainda induzir alterações morfológicas nas plantas, o que afeta as características como área foliar, e razão parte aérea/raiz, o que pode modificar as relações de transpiração e absorção de água pela planta (PIMENTEL, 2004). Em baixa disponibilidade de água, a redução do crescimento da planta é muitas vezes relacionado ao crescimento da raiz como estratégia para melhor absorção de água e redução na produtividade da cultura (LOPES *et al*., 2011). Da mesma forma, é possível relacionar a diminuição da área foliar com o enrolamento das folhas e pela diminuição no crescimento da folha afetada pelo status hídrico do tecido vegetal (MARCOS, 2017). As plantas de cana-de-açúcar, por exemplo, apresentam redução na taxa de crescimento foliar em potencial da água na folha a partir de −0,4 MPa e esta taxa é praticamente zero em −1,3 MPa (LOPES *et al*., 2011). Não obstante, a recuperação do crescimento foliar é rapidamente observada após a reidratação, inclusive superando o crescimento das plantas que não passaram por estresse (INMAN-BAMBER & SMITH, 2005).

Durante o período de estresse, as alterações observadas nas plantas podem, se não sempre, torná-las mais tolerantes a futuras exposições ao déficit hídrico, o que de certa forma pode trazer benefícios para a recuperação das plantas, na fase pós-estresse (WALTER *et al*., 2011). Como exemplo, pode-se mencionar a maior velocidade no fechamento estomático, as alterações anatômicas das folhas de modo a não prejudicar a interceptação de luz, a redução

das perdas de água, o aumento no teor de moléculas osmoprotetoras, e melhoria da eficiência no uso da água. MARCOS (2017) relatou que a cana-de-açúcar é melhorada quando se utilizada colmos da planta mãe que passaram por estresse hídrico, o que pode demonstrar uma memória transgeneracional.

Os efeitos do déficit hídrico sobre o metabolismo C_4 não são tão bem estudados como para o metabolismo C_3, apesar destas plantas C_4 terem relevante importância para captação de CO_2 atmosférico e na alimentação. Existem evidências que o metabolismo C_4 é altamente sensível ao déficit hídrico, e o efeito deste fator do ambiente pode afetar este tipo de metabolismo por meio das limitações estomáticas como também as não estomáticas (GHANNOUM, 2009). Dessa forma, a utilização do modelo C_4 de *S. viridis* torna-se uma ferramenta importante frente aos desafios que a agricultura moderna irá enfrentar nas próximas décadas. O uso dessa planta como modelo, para desvendar alguns dos caminhos que a agricultura deverá tomar, será essencial para o equilíbrio e a segurança alimentar de futuras gerações.

2. OBJETIVO

Utilizar a planta modelo C_4 *Setaria viridis* como plataforma para descrever as respostas:

- ✓ Morfo-anatômicas de plantas submetidas à ação de dois herbicidas (clomazone e saflufenacil); e
- ✓ Morfológicas, fisiológicas e genéticas de plantas submetidas ao déficit hídrico causado por estresse osmótico prolongado.

2.1. Objetivos específicos

- ➢ Para determinação da melhor metodologia para cada tipo de teste (herbicida e osmótico), cultivar o acesso selvagem A10.1 de *S. viridis* em condição de temperatura, irradiância e umidade controladas, e testar diferentes tipos de substratos de cultivo (tipos de solo e sistemas de hidroponia).
- ➢ Em dois tempos (24 e 96 h) após a aplicação dos herbicidas clomazone e saflufenacil, caracterizar a morfologia, anatomia e ultraestrutura de *S. viridis* acesso A10.1.
- ➢ Sob déficit prolongado, escolher a concentração de PEG 8000 e do tempo de exposição ao estresse osmótico, e verificar os efeitos sobre as plantas de *S. viridis*.
- ➢ Para avaliação da resposta de *S. viridis* acesso A10.1 ao estresse osmótico prolongado (10 dias), determinar genes de marcação de seca,
- ➢ Em plantas de *S. viridis* submetidas ao estresse osmótico prolongado, avaliar a eficiência fotoquímica e o conteúdo de prolina.

3. MATERIAL E MÉTODOS

No ano de 2018, foi iniciado o experimento em câmara de crescimento vegetal (tipo Fitotron®) seguido de casa-de-vegetação. Nesse experimento, avaliaram-se os efeitos de diferentes herbicidas na fisiologia, morfologia e anatomia de plantas de *Setaria viridis*. O experimento foi iniciado em 12 de janeiro e finalizado em 8 de fevereiro.

3.1. Crescimento das plantas

O crescimento das plantas foi realizado em câmara de crescimento vegetal, tipo Fitotron®, localizada no Laboratório de Genética Molecular Vegetal (LGMV-UFRJ), na cidade universitária-RJ (22°54'10''S; 43°12'27''W; 5.0 m) (Figura 3). Para simplificação do texto, será utilizado apenas Fitotron. O Fitotron foi configurado para um fotoperíodo de 16/8 h (luz/escuro), e foi utilizada iluminação artificial de 300-400 µmol.m^{-2} s^{-1} com lâmpadas de luz branca. A temperatura da câmara foi controlada com ar condicionado frio/quente e esta variável foi mantida entre 30±2 °C (luz) e 25±2 °C (escuro).

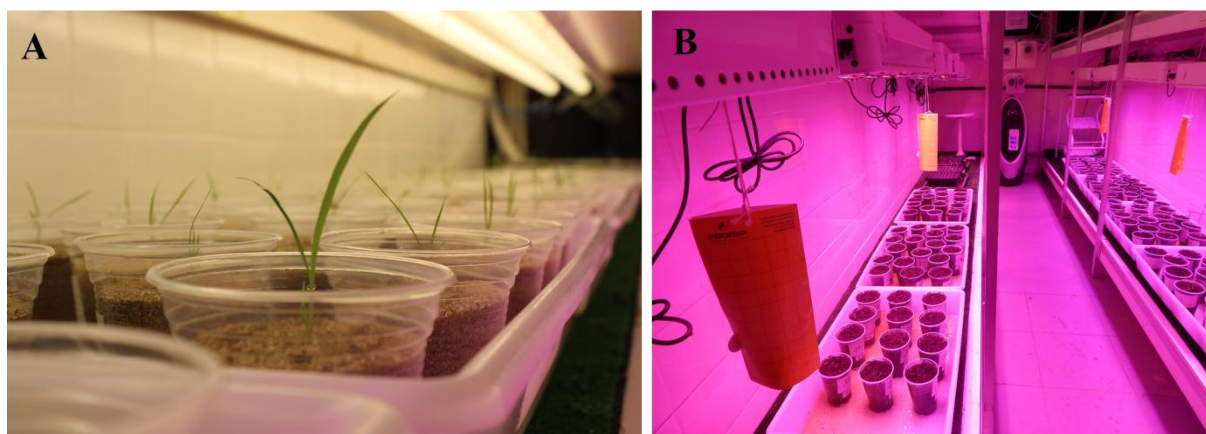

Figura 3: Câmara de crescimento, tipo Fitotron, localizada no Laboratório de Genética Molecular Vegetal (LGMV-UFRJ) com diferentes experimentos.

Sementes de *S. viridis* (acesso A-10.1) foram submetidas à quebra de dormência de acordo com o protocolo descrito para a espécie (SEBASTIAN *et al.*, 2014). Para tanto, foi dissolvido ácido giberélico (GA_3) em etanol 95% para a preparação da solução-estoque a 200 mM e armazenada a -20 ºC. A solução-trabalho (2,89 mM) foi feita dissolvendo o GA_3 em água destilada no momento de uso. Uma solução-estoque de nitrato de potássio (KNO_3) foi dissolvida em água destilada a 500 mM e também mantida a -20 ºC. O tratamento das sementes foi realizado com as sementes submersas em uma mistura 1:1 de GA_3 (2,89 mM) e KNO_3 (30 mM) em eppendorfs® de 2 mL. Aproximadamente 50 sementes foram colocadas

por eppendorf. Após 24 h de submersão, as sementes foram retiradas da solução e lavadas com água destilada três vezes.

A germinação prosseguiu em papel para germinação de sementes com pH neutro (GERMILAB, Cascais, Portugal), e o papel foi colocado em placa de petri com água destilada, e tampada para evitar perda por evaporação. As sementes foram mantidas no Fitotron nas condições supracitas.

Aos 7 dias após a semeadura (DAS), as plântulas de *S. viridis* foram transplantadas para vasos plásticos de 400 mL (8,5 x 11 cm) preenchidos com uma mistura de areia e vermiculita na proporção de 19:1 (m/m, base seca). Cada muda foi acomodada no centro de cada vaso. A mistura recebeu, antes do transplante, 100 mL de uma solução de Hoagland (Caisson labs, nº 2) diluída em água destilada, com pH entre 5,5 e 6,0, NPK (4:1:5). A solução utilizada foi composta por nitrato de potássio [KNO_3] 1.213,2 mg L^{-1}, nitrato de cálcio tetra-hidratado [$Ca(NO_3)_2$, $4H_2O$] 1.889,2 mg L^{-1}, diidrogenofosfato de amônio [$NH_4H_2PO_4$] 460,1 mg L^{-1}, sulfato de magnésio hepta-hidratado [$MgSO_4.7H_2O$] 985,9 mg L^{-1}, ácido bórico [H_3BO_3] 5,7 mg L^{-1}, cloreto de manganês tetra-hidratado [$MnCl_2.4H_2O$] 3,6 mg L^{-1}, sulfato de zinco hepta-hidratado [$ZnSO_4.7H_2O$] 0,4 mg L^{-1}, sulfato de cobre penta-hidratado [$CuSO_4.5H_2O$] 0,2 mg L^{-1}, trióxido de molibdênio [MoO_3] 0,04 mg L^{-1} e sal sódio férrico de ácido etilenodiamino tetra-cético [Fe EDTA] 36,8 mg L^{-1}.

Ao ser emitida a 3ª folha, essa folha foi marcada com fita crepe. Essa marcação é realizada na base da folha, com o objetivo de acompanhar o crescimento das demais folhas, as quais foram emitidas. Os vasos foram regados diariamente e mantidos na capacidade de campo. Aos 20 DAS, as plantas foram transferidas para casa-de-vegetação do grupo de pesquisa Plantas Daninha e Pesticidas no ambiente (PDPA-UFRRJ) em Seropédica, onde os herbicidas foram aplicados. A transferência ocorreu antes do amanhecer e as plantas receberam herbicidas nas primeiras horas da manhã.

3.2. Aplicação dos herbicidas e desenho experimental

A aplicação dos herbicidas ocorreu na casa-de-vegetação localizada no município de Seropédica-RJ (22°48’00”S; 43°41’00”W, 33 m). De acordo com CARVALHO *et al.* (2006), o clima da região é classificado como Aw pela a classificação de Köppen, com chuvas e temperaturas elevadas no verão e um inverno seco com temperaturas amenas. As chuvas ocorrem de novembro a março, com precipitação média anual de 1.213 mm e temperatura anual média de 24,5 °C. Durante a aplicação do herbicida, a temperatura e a umidade relativa

foram de 23,5 °C e 80%. As condições de casa-de-vegetação foram 14 h de dia e temperatura de 25,8 ± 5,9 °C e 10 h de noite a 22,5 ± 2,4 ° C.

A casa-de-vegetação é construída em metal, em a estrutura, envolta por completo com tela tipo sombrite e a cobertura é feita com plástico agrícola de 100 micra. Esta estufa é utilizada para ensaios de herbicidas pelo PDPA-UFRRJ. Durante a realização do experimento, não havia outro ensaio acontecendo.

Dois herbicidas comerciais, clomazone e saflufenacil foram avaliados. As concentrações de herbicidas estudadas foram baseadas em doses comerciais e práticas agrícolas típicas. O delineamento experimental foi inteiramente casualizado e os herbicidas foram testados em quatro concentrações (Tabela 1) e um controle (sem aplicação de herbicida). Cinco repetições por tratamento foram utilizadas. Duas semanas após a semeadura, as plantas com crescimento homogêneo foram selecionadas e distribuídas aleatoriamente para cada tratamento (Tabela 1).

Tabela 1: Herbicidas e as respectivas concentrações da dose comercial aplicada sobre *Setaria viridis*.

Nome comum	**Nome comercial**	**Composição do herbicida (g ia L^{-1})**	**Concentrações (g ia ha^{-1})**			
			0.5D	**D**	**1.5D**	**2D**
		Clomazone – Inibidor da biossíntese de carotenoides				
Clomazone	Gamit	500	500	1000	1500	2000
		Saflufenacil – inibidor de PPO				
Saflufenacil	Heat®	-	49	98	147	196

1. D= dose comercial; ia= ingrediente ativo; *PPO: protoporfirinogênio IX oxidase

O herbicida foi aplicado por volta das 9 horas da manhã, e foi utilizado um pulverizador do tipo mochila pressurizada com CO_2 a 280 kPa, e com quatro bicos de ventilador plano XR-110015 (TeeJet Technolodies, Wheaton, IL). Nesta condição, foi fornecido 200 L ha^{-1} (Figura 4).

Figura 4: Aplicação do herbicida (A) e vasos com plantas dispostas na casa-de-vegetação após a aplicação (B).

3.3. Avaliação de injurias foliares

Nos horários de 24, 96 e 168 h após a aplicação do herbicida (AAH), e de acordo com os níveis de lesão (entre clorose, necrose e murcha), as lesões nas folhas das plantas foram classificadas visualmente entre 0 (sem lesões) a 100 (lesões graves) (FRANS, 1986). Após as avaliações das lesões, foram obtidas fotografias das plantas usando uma câmera digital Canon EOS Rebel T6-S, tanto nas plantas que receberam a dose comercial de cada herbicida, como as plantas controle. Posteriormente, essas fotografias foram classificadas da seguinte forma: fase inicial = início das lesões, lesões leves a moderadas; fase intermediária = lesões moderadas a graves; e fase final = lesões graves até a morte.

3.4. Determinação do conteúdo de clorofilas *a* e *b* e carotenoides

Para a determinação do conteúdo de pigmentos fotossintéticos (clorofilas *a* e *b* e carotenoides), foram coletadas amostras de folhas de cinco plantas em cada tratamento às 24 e 96 h AAH. Apenas as plantas que receberam a dose comercial de cada herbicida e as plantas controle foram avaliadas. Para extração destes pigmentos, utilizou-se dimetilsulfóxido (DMSO) como descrito por SHINANO *et al*. (1996). Cerca de 20-50 mg de folhas (massa fresca) foram incubados em tubos de vidro de cor âmbar contendo 2 mL de DMSO. A extração ocorreu por aquecimento em banho-maria a 65 ° C por 60 minutos. O extrato foi filtrado e a absorvância da alíquota foi analisada em um espectrofotômetro Libra S22 - Biochrom, (Inglaterra) nos comprimentos de onda de 480, 649 e 665 nm. O conteúdo das clorofilas *a* e *b* e dos carotenoides foram calculados pelas seguintes equações (BARNES *et al*., 1992).

$$\text{Clorofila } a \left(\mu g\ ml^{-1}\right) = 12.47 \text{ x} A_{665} - 3.62 \text{ x } A_{649}$$

$$\text{Clorofila } b \ (\mu g\ ml^{-1}) = 25.06 \text{ x } A_{649} - 6.50 \text{ x } A_{665}$$

$$\text{Carotenoides } (\mu g\ ml^{-1}) = \frac{1000 \text{ x } A_{480} - 1.29 \text{ Chl } a - 53.78 \text{ Chl } b}{220}$$

3.5. Análises anatômicas, micromorfológicas e ultraestruturais

Às 24 e 96 h da AAH, as lâminas foliares do quinto e sexto nós foram cortadas da terceira seção média das plantas que receberam a dose comercial de cada herbicida e as plantas controle. Três seções de cinco repetições por tratamento foram obtidas e imediatamente fixadas em 4% de paraformaldeído e 2,5% de glutaraldeído em solução tampão de cacodilato 0,1 M. As amostras fixadas foram incubadas a 4 ºC durante a noite. As amostras foram pós-fixadas em 1% de tetróxido de ósmio em soluções tampão de cacodilato 0,1 M por 1 h.

Para análise anatômica (LM), as folhas fixadas foram desidratadas em uma série de etanol, embebidas na resina de Spurr e polimerizadas a 70 °C durante a noite. Cortes finos transversais (1 µm) foram cortados usando um ultramicrótomo PowerTome-PC (RMC Products, Tucson, AZ, EUA), corado com azul de toluidina a 0,5% e fotografado com um microscópio óptico de campo claro (Leica DM 2500-Wetzlar, Alemanha) no Núcleo de Pesquisa Multidisciplinar (NUMPEX-Bio) da Universidade Federal do Rio de Janeiro, Estado do Rio de Janeiro, Brasil. A espessura da lâmina foliar, da superfície abaxial à adaxial, foi obtida de quatro fotomicrografias com ampliação de 20X (dez medidas foram realizadas em cada fotomicrografia, totalizando 40 medidas por tratamento), utilizando o software ImageJ (NIH).

Para a análise micromorfológica (SEM), as amostras de folhas foram desidratadas em séries etanólicas até 100% e secas até o ponto crítico (K850 Critical Point Dryer, Quorum Technologies). As amostras foram então colocadas em tocos de pinos com fita de carbono e revestidas com ouro (Desk V Sample Preparation, Denton Vacuum). As observações e fotografias foram obtidas em microscópio eletrônico de varredura (Vega 3LMU Tescan, Brno, República Tcheca) também no NUMPEX-Bio. Posteriormente, a área do estoma e o comprimento dos poros foram medidos usando o software ImageJ (NIH) de 20 fotografias SEM.

Para análise ultraestrutural (TEM), as seções ultrafinas (60 nm) foram cortadas em um ultramicrótomo (RMC Products, Tucson, AZ, EUA), coradas com 1% de acetato de uranil,

por 25 minutos e citrato de chumbo por 3 minutos. As amostras foram observadas em microscópio eletrônico de transmissão (TEM) (FEI - TECNAI SPIRIT 80kV) e as imagens foram adquiridas pelo programa TEM Imaging Platform no Instituto Nacional de Metrologia, Qualidade e Tecnologia (Inmetro / RJ) e CENABIO (UFRJ).

3.6. Análises estatísticas dos dados

O conteúdo de pigmento fotossintético (clorofilas *a* e *b* e carotenoides) e as medidas anatômicas foram realizadas em cinco repetições biológicas. Diferenças significativas entre médias foram avaliadas pelos testes Tukey-Kramer HSD no nível $P \leq 0{,}05$ no software StatSoft (versão 8.0). A distribuição normal dos dados e a homogeneidade das variâncias foram verificadas com os testes de Cochran C. Hartley, Bartlett, respectivamente, antes de serem submetidos à análise de variância (ANOVA).

No ano de 2019, foi iniciado um experimento no Fitotron para avaliar os efeitos do estresse osmótico na fisiologia, morfologia e expressão gênica de plantas de *Setaria viridis*. O experimento foi realizado em réplica, sendo o primeiro iniciado em 8 de outubro e finalizado em 6 de novembro, e o segundo iniciado e finalizado em 29 de outubro e 27 de novembro, respectivamente.

3.7. Crescimento das plantas

O crescimento das plantas foi realizado no mesmo Fitotron descrito no item 3.1 (Figura 3). Para tanto, foi usada a mesma configuração de fotoperíodo e temperaturas; ou seja de 16/8 h (luz/ escuro), 30±2 °C (luz) e 25±2 °C (escuro). No entanto, para este experimento foi utilizado iluminação artificial de 300-400 $\mu mol.m^{-2}\ s^{-1}$ com lâmpadas de luz branca para a germinação e de 750 $\mu mol.m^{-2}\ s^{-1}$ com lâmpadas LED's de composição vermelho, azul, branco e laranja para o crescimento das plantas.

Cerca de quatro a cinco sementes de *S. viridis* (acesso A-10.1) foram colocadas sobre areia dentro de gargalos de garrafas PET cortados e virados ao contrário (Figura 3). No fundo de cada gargalo cortado, foram colocados pedaços de tule amarrados com elástico, para evitar que a areia saísse, ao passo que permitia o crescimento das raízes. Esses conjuntos foram colocado sobre tampas de potes de 1,5 – 2 L de sorvete ("berçários") inseridos em furos que permitiam o contado do fundo das tampas com o interior dos potes. Foram colocados de sete a oito gargalos por tampa (Figura 5). Esses potes foram então preenchidos com água deionizada até um volume que permitisse que a areia se mantivesse sempre úmida. À medida que as germinações ocorriam, as sementes que sobravam nos gargalos eram retiradas, deixando apenas uma plântula por gargalo.

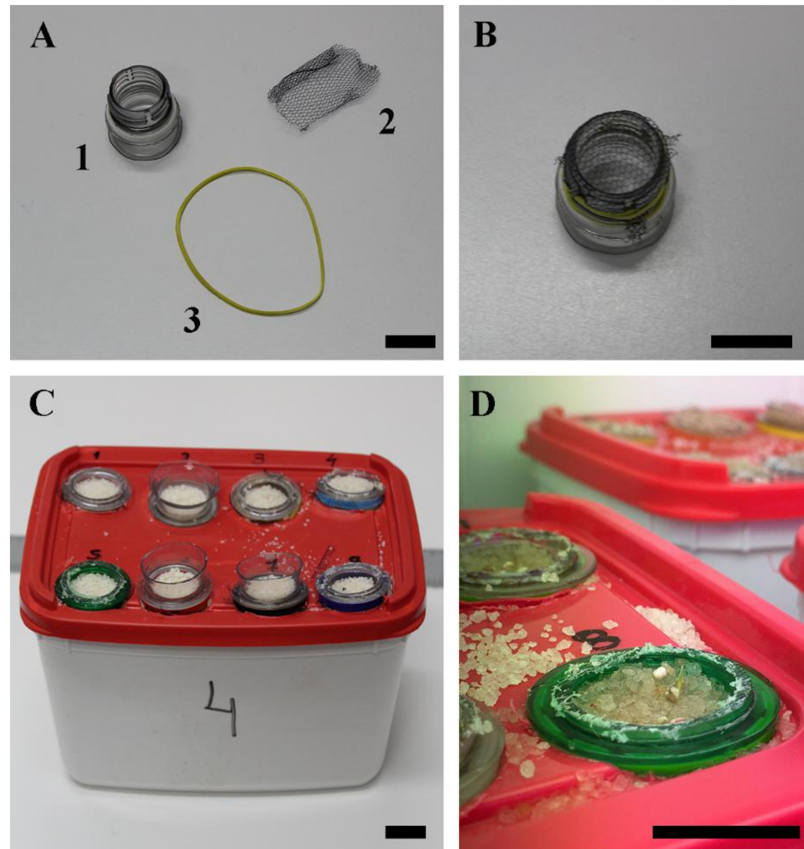

Figura 5: Montagem do “berçário” para germinação das sementes de *Setaria viridis*. Componentes: 1. Gargalos de garrafa PET; 2. Tulle; e 3. Elástico. Barras de escala: 2 cm.

Após 7 dias, quando as plântulas estavam com a folha do segundo nó completamente expandida, cada gargalo era transferido para o sistema de hidroponia individual sem aeração (Figura 6). Esse sistema consiste no conjunto de um copo plástico transparente, inserido em um copo plástico de cor preta, ambos com volume de 200 mL. A solução nutritiva é colocada no copo transparente, que permite a visualização das raízes, enquanto que o copo preto bloqueia a passagem de luz, evitando o crescimento de microalgas na solução. Na parte superior do copo, é colocada uma tampa de cor preta com um furo no centro para a inserção do gargalo de garrafa PET contendo a plântula. Todo esse conjunto era envolvido com papel alumínio para evitar o aquecimento da solução por indução (Figura 6).

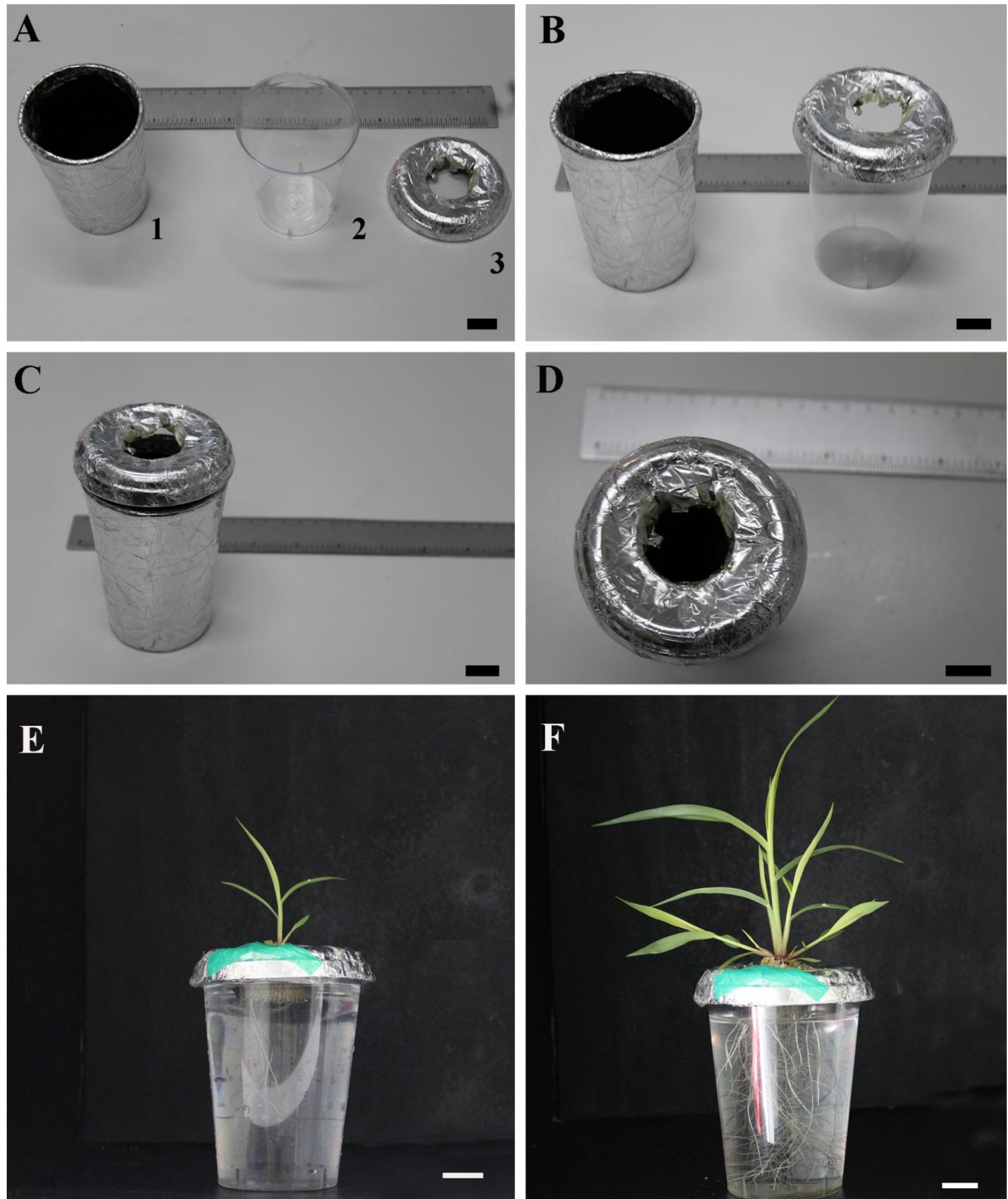

Figura 6: (A-D) Montagem do sistema hidropônico individual. Componentes: 1. Copo plástico preto (200 mL) envolvido por papel alumínio; 2. Copo plástico transparente (200 mL); e 3. Tampa plástica preta envolvida por papel alumínio cortada no centro para inserção de gargalo de PET. (E-F) Sistema de hidroponia montado com as plantas apoiadas acima das tampas com as raízes em solução (imagens sem o componente 1). Barras de escala: 2 cm.

Neste experimento, foi utilizada uma solução nutritiva na concentração de 25% de Hoagland (Caisson labs, nº 2), diluído em água destilada. A troca da solução era realizada a cada 7 dias, e diariamente era acrescentado água destilada para repor volume de água perdido

por transpiração. A última troca de solução foi realizada por concentração de 50% de Hoagland (Caisson labs, nº 2). A solução nutritiva nessas concentrações (25/50%) foi composta por nitrato de potássio [KNO_3] 152/ 303 mg L^{-1}, nitrato de cálcio tetra-hidratado [$Ca(NO_3)_2$, $4H_2O$] 236/ 472 mg L^{-1}, diidrogenofosfato de amônio [$NH_4H_2PO_4$] 58/ 115 mg L^{-1}, sulfato de magnésio hepta-hidratado [$MgSO_4.7H_2O$] 123/ 246 mg L^{-1}, ácido bórico [H_3BO_3] 0,72/ 1,4 mg L^{-1}, cloreto de manganês tetra-hidratado [$MnCl_2.4H_2O$] 0,45/ 0,9 mg L^{-1}, sulfato de zinco hepta-hidratado [$ZnSO_4.7H_2O$] 0,05/ 0,1 mg L^{-1}, sulfato de cobre penta-hidratado [$CuSO_4.5H_2O$] 0,02/ 0,04 mg L^{-1}, trióxido de molibdênio [MoO_3] 0,005/ 0,01 mg L^{-1} e sal sódio férrico de ácido etilenodiamino tetra-cético [Fe EDTA] 4,6/ 9,2 mg L^{-1}.

Após 7 dias da transferência ao sistema de hidroponia, período de aclimatação, quando as plantas estavam com idade de 15 dias e com a folha do quarto nó completamente expandida, foram aplicados os tratamentos.

3.8. Tratamentos e desenho experimental

Os tratamentos aplicados foram com a utilização ou não de 7,5 % [g mL^{-1}] de polietilenoglicol de peso molecular de 8000 (PEG-8000, aproximadamente -0,09 MPa). A obtenção dessa concentração foi obtida dissolvendo-se 7,5 g de PEG-8000 a cada 100 mL de solução de Hoagland. O tratamento controle continha apenas solução de Hoagland, sem a dissolução de PEG. Cada pote contendo uma planta representava uma parcela experimental, e eram dispostas aleatoriamente no espaço experimental (Fitotron). A cada dois dias, a posição das parcelas experimentais (cada pote com uma planta) era randomizada para evitar discrepâncias naturais ao posicionamento no ambiente experimental. Os tratamentos seguiram por 10 dias e as análises foram realizadas ao longo do experimento, conforme será mostrado adiante. Para simplificação, os dias após a aplicação dos tratamentos (DAAT) serão chamados "dia 1" a "dia 10", sucessivamente. Esse experimento foi realizado em réplica, utilizando-se as mesmas condições experimentais.

3.9. Fluorescência da clorofila *a*

A emissão da fluorescência da clorofila *a* foi mensurada em folhas, do último nó contado a partir da base, completamente expandidas nos dias 3, 6 e 10 DAAT. As análises foram realizadas em dez plantas por tratamento (controle e PEG-800), em horário próximo às 8 h da manhã (2 h após o início do ciclo de 16 h de luz). As medidas foram realizadas utilizando-se um fluorômetro portátil (HandyPEA, Hanstech, King's Lynn, Norkfolk, UK). As folhas utilizadas foram adaptadas ao escuro por 20 minutos antes das avaliações. A

emissão de fluorescência foi induzida em uma área de 4 mm^2 de folha exposta a um pulso de luz saturante (3000 µmol m^{-2} s^{-1}) por 0,8 s.

A partir da indução da curva transitória de fluorescência, obtida após o pulso de saturação, foram armazenadas as intensidades da fluorescencia em 50 µs (fluorescência inicial - F_O), 100, 300 µs, 2 (F_J) e 30 (F_I) ms e F_M (fluorescência máxima). A partir da emissão nestes tempos em escala do eixo x logaritmizadas, foi possivel obter as variáveis estabelecidas pelo teste JIP (YUSUF *et al.*, 2010). Além disso, o teste JIP foi aplicado para a análise e comparação dos transientes OJIP, usando as normalizações e as subtrações. Ainda, a cinética da diferença dos dados relativos de fluorescência variável também foi calculada de acordo com Yusuf *et al*. (2010). Os transientes foram normalizados (símbolo geral W) como variável relativa fluorescente para comparar as amostras para os eventos refletidos nas fases OJ, OI e IP como $W_{OJ} = (F_t - F_0) / (F_J - F_0)$, $W_{OI} = (F_t - F_0) / (F_I - F_0)$ e $W_{IP} = (F_t - F_I) / (F_P - F_I)$. Dessa forma, a cinética da diferença que revela bandas geralmente ocultas entre as etapas O, J, I e P dos transientes brutos ou normalizados foi calculada como $\Delta W = W - W_{ref}$ ("ref" é usado para o controle).

3.10. Análises biométricas e condutância estomática

Em cada tratamento todos os dias, e ao longo do experimento, as análises morfológicas (número de folhas total; número de folhas do caule principal; número do perfilho; e ângulo de exposição da última folha; este último medido com um transferidor) foram realizadas a partir de dez plantas. Aos 6 e 10 DAAT, foram avaliados a massa seca da parte aérea e raiz; o conteúdo relativo de água das folhas e das raízes; o extravasamento de eletrólitos (*leakage*) da parte aérea e da raiz; a área foliar; o potencial osmótico de folhas e das raízes; e o conteúdo de prolina nas folhas e nas raízes. As folhas de cinco plantas por tratamento foram colocadas em papel branco e fotografadas para medição da área total, usando o software ImageJ (NIH). Os dias de amostragem foram escolhidos com base em um experimento piloto, em que as plantas de *S. viridis* apresentaram efeitos severos de estresse no dia 6 e sensível recuperação no dia 10 DAAT em algumas variáveis relacionadas à fotossíntese (dados não são mostrados).

A condutância estomática (g_s) foi medida em ambas as superfícies foliares (adaxial e abaxial) usando um porômetro foliar (SC-1, Decagon Devices, Washington, EUA). As medições de g_s foram realizadas todos os dias, próximo às 9 h da manhã (3 horas após o sistema de iluminação ser ligado) (ANEXO III).

3.11. Conteúdo relativo de água (CRA)

Aos 6 e 10 DAAT, as medidas de conteúdo relativo de água (CRA) seguiram a metodologia descrita por VALENÇA et al. (2018). As folhas intactas dos últimos nós, totalmente expandidas e a parte de raízes foram coletadas de cinco plantas em cada tratamento. Cada tecido foi pesado separadamente logo após a coleta, caracterizando-se a massa fresca (MF). Imediatamente após a pesagem, os tecidos foram imersos em água destilada, dentro de tubos tipo Falcon, por 24 h (a 3 °C) para posterior determinação massa túrgida (MT). Para a obtenção da MT, antes da pesagem os tecidos foram suavemente secos em papel absorvente. Depois disso, as folhas e raízes foram secas em um forno de ar forçado a 65 °C até atingir um massa constante (PS - aproximadamente 72 h em estufa). O CRA foi calculado usando a fórmula CRA = (MT - MF) / (MT - MS) × 100.

3.12. Extravasamento de eletrólitos (*leakage*)

Aos 6 e 10 DAAT, a análise do extravasamento celular de eletrólitos (*leakage*) seguiu a metodologia descrita por LIU *et al*. (2015). As folhas frescas e tecido de raiz (0,2 g), de cinco plantas por tratamento, foram colocados separadamente em tubos do tipo Falcon contendo 20 mL de água deionizada. As amostras foram mantidas em um agitador durante a noite e, em seguida, a condutividade elétrica da solução (C1) foi medida. Logo após, as amostras foram auto clavadas a 120 °C por 30 min e, quando os tubos atingiram a temperatura ambiente, a condutividade da solução com o tecido foi medida novamente (C2). O *leakage* foi calculado usando a equação: EL (%) = (C1/C2) x100. A condutividade elétrica da solução (C1 e C2) foi medida usando um condutivimetro modelo 3540, Jenway, Staffordshire, Reino Unido.

3.13. Medição do potencial osmótico

Aos 6 e 10 DAAT, o potencial osmótico (Ψ_{osm}) das folhas e das raízes foi medido usando um medidor de potencial de ponto de orvalho (WP4C, Decagon Devices). As amostras foram coletadas e congeladas imediatamente por imersão em nitrogênio líquido e depois colocadas em congelamento a -80 ° C para posterior análise. As medições foram feitas em folhas e raízes colocadas na câmara WP4C e imediatamente seladas (LONDERS *et al*., 2005). As amostras foram organizadas de forma que o fundo da câmara estivesse totalmente coberto. Três repetições foram realizadas, e a média aritmética das leituras foi considerada como o valor do Ψ_{osm}. O instrumento foi calibrado de acordo com as recomendações da

Decagon Devices com solução de KCl (KCl 0,5 M; Ψ = -2,19 MPa). A calibração foi repetida no início de cada dia de leitura, quando eram realizadas de três a quatro leituras.

3.14. Conteúdo de prolina

Aos 6 e 10 DAAT, o conteúdo de prolina livre foi determinado com base no método de ZHANG e HUANG (2013). As amostras de plantas (folhas e raiz; 0,2-0,25 g) foram moídas com nitrogênio líquido e homogeneizadas com 2 mL de ácido sulfossalicílico a 3%. O extrato obtido foi centrifugado a 5.000 g por 5 min. Logo após, em um tubo de ensaio rosqueável, foi adicionado 500 µL do sobrenadante a 500 µL de ninhidrina ácida (2,5 g de ninhidrina ácida; 60 mL de ácido acético glacial e 40 mL de ácido fosfórico 6 M) e 500 µL de ácido acético. A mistura resultante foi fervida por 45 min em banho-maria e depois resfriada em gelo por 30 min. Depois, foi adicionado 1.500 µL de tolueno à mistura e centrifugado a 1.000 g por 5 min. A densidade óptica da solução sobrenadante foi medida a 520 nm usando um espectrofotômetro Libra S22 - Biochrom, (Inglaterra). Uma curva de calibração foi preparada com concentrações conhecidas de prolina (1; 10; 50; 100; 150; 200; 300 µM) para determinar o conteúdo de prolina livre como: $\mu M_{(prolina)}\ g_{(amostra)}^{-1}$.

3.15. Análise de dados

Os dados fisiológicos foram analisados pelo software Sisvar (Universidade Federal de Lavras, Lavras, Brasil). Os resultados médios de duas repetições experimentais foram submetidos à análise de variância ($p \leq 0,05$), seguida de teste t pareado para determinar diferenças significativas em cada data de amostragem entre plantas controles e tratadas usando PEG 8000. Foi realizada uma Análise de Componentes Principais (PCA) usando características biométricas, condutância estomática e parâmetros fotoquímicos para investigar agrupamentos variáveis pelo software PAST, da versão 3 (PAleontological STatistics).

3.16. Análise de expressão gênica

Para análise da expressão gênica, as raízes das plantas de *S. viridis*, de ambos tratamentos, foram coletadas nos dias 3, 6 e 10 DAAT. As amostras foram armazenadas a -80 °C e posteriormente moídas em nitrogênio líquido. As raízes de três plantas foram moídas em conjunto para formar uma réplica biológica e o procedimento foi repetido três vezes para obter três réplicas biológicas. Para extração de RNA, utilizou-se o tecido macerado (0,15 g) usando o Sistema de Isolamento de RNA Total SV da Promega®. A concentração e pureza das amostras de RNA foram medidas usando Nanodrop 2000 (Thermo Scientific). A síntese

da cadeia de DNA complementar (cDNA) foi realizada usando 2 μg de RNA total, adicionando 50 μM de Oligo dT primer, 10 mM de dNTPs, tampão First-Strand 5X, 20 mM de DTT e 200 unidades da enzima SuperScriptTM III (Invitrogen), de acordo com as instruções do fabricante. O cDNA foi armazenado a -20 °C para ser posteriormente usado em análises de qPCR.

Os genes escolhidos para análise da expressão foram selecionados com base em estudos anteriores realizados pelo nosso grupo. O primeiro estudo identificou genes marcadores de seca em *S. viridis* expostos a duas condições diferentes de déficit hídrico (RODRIGUES *et al*., 2020); enquanto o segundo estudo utilizou a análise do transcriptoma de raízes de *S. viridis* expostas a ciclos de desidratação, nos quais foi possível identificar os genes de resposta tardia (RODRIGUES *et al*., dados não publicados). Com base nesses dois estudos, selecionamos cinco genes a serem avaliados pelo qPCR. Os primers foram projetados com uma temperatura de recozimento de 60 °C, e foi usada a ferramenta Primer3Plus (UNTERGASSER *et al*., 2007). As sequências iniciadoras e os códigos dos genes estão listados na Tabela Suplementar S1 (artigo 2). O PCR quantitativo em tempo real (qPCR) foi realizado usando o sistema de detecção de PCR em tempo real 7500 Fast (Applied Biosystems), polimerase de DNA PlatinumTM Taq (Invitrogen) e SYBR Green como corante para monitorar a amplificação do DNA. O corante de referência ROX (Invitrogen) foi usado para a correção dos dados da amostra com base na fluorescência de fundo. Os dados brutos de amplificação foram analisados usando o PCR em tempo real (ZHAO & FERNALD, 2005) para identificar a fase exponencial da reação e calcular o Ct (limiar do ciclo). A expressão gênica relativa foi calculada usando a Relative Expression Software Tool - 384 (REST - 384) (Pfaffl et al., 2002), comparando amostras de controle e tratadas e testando a significância com um Teste de Aleatorização de Realocação Fixa Pair Wise. Dois genes constitutivos (Seita.1G130900 e Seita.3G037700), previamente identificados como bons genes de referência durante o déficit hídrico por estresse osmótico, foram utilizados para o cálculo do fator de normalização. Os resultados relativos da expressão gênica são apresentados como log2Fold-Change (log2FC), em que a alteração dobrada é a razão entre os níveis de expressão das amostras tratadas e de controle. Os resultados foram plotados em um mapa de calor, construído em R usando o pacote latticeExtra (SARKAR & ANDREWS, 2016)

4. CAPÍTULO I – ARTIGO I

Changes in Leaf Blade Morphology and Anatomy Caused by Clomazone and Saflufenacil Observed in *Setaria viridis* a Model C_4 Plant

Artigo publicado no periódico South African Journal of Botany (SSN 0254-6299) sob o número de DOI:10.1016/j.sajb.2020.09.027 (ANEXO I)

Changes in Leaf Blade Morphology and Anatomy Caused by Clomazone and Saflufenacil in *Setaria viridis*, a Model C_4 Plant

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ABSTRACT

Clomazone and saflufenacil are herbicides extensively used worldwide to weed control. We studied the effects of these two herbicides on morphoanatomical parameters of *Setaria viridis*. Plants were sprayed with four concentrations of each herbicide (clomazone: 500, 1000, 1500 and 2000 g of active ingredient (ai) ha^{-1} and saflufenacil: 49, 98, 147 and 196 g ai ha^{-1}) besides control (without spraying) 20 days after transplantation. The experimental design was completely randomized with five replicates per treatment. Pigment content, visible injuries, morphological and ultrastructural changes were evaluated. No signs of tolerance to either of the tested herbicides were observed. Clomazone caused a decrease in photosynthetic pigment content over time, mostly in young leaves, leading to an “albino” like appearance. There was a reduction in the number of grana in the chloroplasts of mesophyll cells (MC) in necrotic

areas. Saflufenacil reduced chlorophyll content, impairing energy absorption in the antenna complex. Injuries to foliar tissues, such as necrosis and depigmentation, were visible as early as 24 h after herbicide application. Bundle sheath chloroplasts (BSC) and MC were completely deformed. The data support the use of *S. viridis* as a model plant for studies on herbicide effects in C_4 monocots.

Key words: foliar surface - C_4 photosynthesis- ultrastructure - visible injury - Poaceae.

1. Introduction

Weed infestation is responsible for about 34% of yield reductions of major crops around the world (Li *et al.*, 2017). Intensive modern agriculture depends widely on pesticides to guarantee high productivity (Du *et al.*, 2018). Clomazone [2 - [(2 - chlorophenyl) methyl] - 4,4 - dimethyl - 1,2 - oxazolidin - 3 - one] and saflufenacil (N' - {2 - chloro - 4 - fluoro - 5 - [1,2,3,6 - tetrahydro - 3 - methyl - 2,6 - dioxo - 4 - (trifluoromethyl) pyrimidin - 1 - yl] benzoyl} - N - isopropyl - N - methylsulfamide) are two herbicides largely used worldwide and their consumption continues to increase.

Clomazone is a member of the isoxazolidinone chemical group, classified as a carotenoid and a chlorophyll biosynthesis inhibitor (Senseman, 2007) and used as a selective herbicide for many crops such as paddy field rice, maize and sugarcane to control annual Poaceae and broadleaf weeds (Cabral *et al.*, 2017). Clomazone treated seedlings of susceptible species are usually bleached white, yellow or reddish purple before death (Duke *et al.*, 1985; Duke and Kenyon, 1986). Tolerant species show transient and minor symptoms, such as chlorosis along the margins of older leaves.

Saflufenacil belongs to the pyrimidinedione family of herbicides and is a protoporphyrinogen IX oxidase (PPO) inhibiting herbicide with foliar and soil activity (Grossmann *et al.*, 2010). Plants susceptible to saflufenacil herbicide exhibit typical injuries that include tissue necrosis along with growth impairment, which often culminates in plant death (Grossman *et al.*, 2011; Reddy *et al.*, 2014). Saflufenacil was registered in the US for broadleaf weed control for preplant burndown and preemergence control for several crops including maize, grain sorghum, rice, and for crop desiccation (Grossmann *et al.*, 2011).

The mechanism of action of these herbicides is well established in the literature, while damage to plant tissues needs to be better described (Cabral *et al.*, 2017; Grossmann *et al.*, 2010). We chose *Setaria viridis* (L.) P. Beauv., a model plant, to evaluate the effects of clomazone and saflufenacil on leaf blade morphology and anatomy. This species belongs to

the subfamily Panicoideae (Poaceae) and is widely spread worldwide as result of its high environmental adaptability (Bennetzen *et al*., 2012). *Setaria viridis* is being studied as a model plant for C_4 photosynthesis due to a number of characteristics that facilitate scientific research; including small genome (approximately 515Mb), short life cycle (approximately 60d) and small stature (10-40cm) (Li and Brutnell, 2011). Additionally, its close evolutionary relationship with Panicoideae crops, such as sorghum and maize, contributes to its establishment as an efficient model plant (Li and Brutnell, 2011; Muthamilarasan and Prasad, 2015).

Here, we present data on the leaf blade structure and the photosynthetic pigment content of plants subjected to clomazone and saflufenacil herbicides for 24 and 96 h. These data will provide valuable data in understanding the limits of plant plasticity, and represents a contribution to the establishment of *S. viridis* not only as a C_4 model plant, but also as a platform for studies of the effects of herbicides in grasses and other monocots.

2. Materials and methods

2.1. Plant and growth conditions

This study was conducted in Rio de Janeiro, State of Rio de Janeiro, Brazil (22°54'10''S; 43°12'27''W; 5.0 m). Seeds of *S. viridis* A10.1 accession were submerged in a 1:1 mixture of GA_3 (2.89 mM) and KNO_3 (30 mM) in a 0.002 L Eppendorf tube, following the protocol of dormancy breaking described by Sebastian *et al.* (2014). After 24 h seeds were placed in petri dishes on germitest® paper moistened with distilled water until the first leaf was fully expanded (six days post imbibition - DPI).

Seedlings were individually transplanted to 0.4 L pots filled with sand and crushed vermiculite mix at a 19:1 ratio (*w*/*w*; dry base). Each pot was watered once with 0.1 L of a homemade Hoagland solution equivalent to the commercial solution nº 2 (Caisson Labs). Petri dishes and pots were maintained in a growth chamber under 16 h of light (300-400 µmol m^{-2} s^{-1}) and a temperature of 32±2 °C, followed by 8 h of dark at 24±2 °C. Pots were watered daily to field capacity and at 20 DPI plants were transferred to a greenhouse at predawn, where the herbicides were applied.

2.2. Experimental design and herbicide application

Two commercial herbicides were evaluated, clomazone and saflufenacil. The concentrations of herbicides that were studied were based on commercial doses and typical agricultural practice. The experimental design was completely randomized and the

herbicides were tested at four concentrations (Table 1) and a control (no herbicide application). Five replicates were used per treatment. Two weeks after sowing, plants with homogeneous growth were selected and randomly assigned for each treatment (Table 1).

Herbicide was applied around 9 a.m. using a CO_2 pressurized backpack sprayer at 280 kPa with four XR-110015 flat fan nozzles (TeeJet Technolodies, Wheaton, IL) delivering 200 L ha^{-1}. During herbicide application, temperature and relative humidity were 23.5 °C and 80%, respectively. Greenhouse conditions were 14 h of daytime and temperature of 25.8±5.9 °C and 10 h of night at 22.5±2.4 °C.

2.3. Leaf injuries

For the analyses of injuries, leaves of each plant were classified visually between 0 (without injuries) to 100 (severe injuries) according to the level of injury (chlorosis, necrosis and wilt) by the following times: 24, 96 and 168 h after herbicide application (AHA). This classification followed the method of Frans (1986). Photographs were taken from plants that received the commercial dose of each herbicide treatment plus control using a Canon EOS Rebel T6-S digital camera. Later, these photographs were classified as following: (i) initial phase: start of injuries, light to moderate injuries; (ii) intermediary phase: moderate to severe injuries; and (ii) final phase: severe injuries to death (Figure 1).

2.4. Chlorophyll and carotenoids content

Pigment contents were extracted at 24 and 96 h AHA using the dimethyl sulphoxide (DMSO) method as described by Shinano *et al.* (1996). Samples were collected from plants that received the commercial dose of each herbicide plus the control plants. Five replicates per treatment were taken for this analysis. Fresh tissues were incubated in glass tubes at 65 °C for 60 min prior to extraction. Absorbance at 480, 649 and 665 nm was determined by a spectrophotometer *Libra* S22 - Biochrom, (England). Contents of chlorophylls *a* and *b* and carotenoids were calculated by the following equations (Barnes *et al.*, 1992).

$$\text{Chl } a \left(\mu\text{g ml}^{-1}\right) = 12.47 \text{ x} A_{665} - 3.62 \text{ x } A_{649}$$

$$\text{Chl } b \left(\mu\text{g ml}^{-1}\right) = 25.06 \text{ x } A_{649} - 6.50 \text{ x } A_{665}$$

$$\text{Carotenoids} \left(\mu\text{g ml}^{-1}\right) = \frac{1000 \text{ x } A_{480} - 1.29 \text{ Chl } a - 53.78 \text{ Chl } b}{220}$$

2.5. Anatomical, micromorphological and ultrastructural analyses

For anatomical analysis (LM), leaves fixed were dehydrated in an ethanol series, embedded in Spurr's resin, and polymerized at 70 °C overnight. Transverse thin sections (1

µm) were cut using an ultramicrotome PowerTome-PC (RMC Products, Tucson, AZ, USA), stained with 0.5 % toluidine blue and photographed with a bright field optical microscope (Leica DM 2500-Wetzlar, Germany) also at the NUMPEX-Bio. Leaf blade thickness, from abaxial to adaxial surfaces was obtained from four photomicrographs at 20X magnification (ten measures were performed in each photomicrographs, totalizing 40 measures per treatment) using ImageJ (NIH) software.

For micromorphological (SEM) analysis, leaf samples were dehydrated in ethanolic series up to 100%, and dried to the critical point (K850 Critical Point Dryer, Quorum Technologies). Samples were then placed on pin stubs with carbon tape and sputter-coated with gold (Desk V Sample Preparation, Denton Vacuum). Observations and photographs were obtained using a scanning electron microscope (Vega 3LMU Tescan, Brno, Czech Republic) at the Nucleus of Multidisciplinary Research (NUMPEX-Bio) at Federal University of Rio de Janeiro, State of Rio de Janeiro, Brazil. Later, the stoma area and pore length were measured using ImageJ (NIH) software from 20 SEM photographs.

The ultrathin sections (60 nm) were cut in an ultramicrotome (RMC Products, Tucson, AZ, USA), stained with 1% uranyl acetate, during 25 min, and lead citrate for 3 min. Samples were observed using a transmission electron microscope (TEM) (FEI - TECNAI SPIRIT 80kV) and images were acquired by TEM Imaging Platform program at National Institute of Metrology, Quality and Technology (Inmetro/ RJ) and CENABIO (UFRJ).

For ultrastructural analysis (TEM), at 24 and 96 h AHA, leaf blades from the fifth and sixth nodes were cross-sectioned from the middle third section of plants that received the commercial dose of each herbicide plus control plants. Three sections of five replicates per treatment were taken and immediately fixed in 4% of paraformaldehyde and 2.5% of glutaraldehyde in 0.1 M cacodylate buffer solution. Fixed samples were incubated at 4 ºC overnight. Samples were post-fixed in 1% of osmium tetroxide in 0.1 M cacodylate buffer solutions for 1 h.

2.6. Statistical analysis

Photosynthetic pigment content, micromorphological and anatomical measurements were carried out in five biological replicates. Significant differences between means were evaluated by Tukey-Kramer HSD tests at $P \leq 0.05$ level in StatSoft (version 8.0) software. The normal distribution of the data and the homogeneity of variances were verified with the Cochran's C. test and Bartlett's tests, respectively, before being subjected to analysis of variance (ANOVA).

3. Results

3.1. Visible injuries

Control plants showed no signs of injuries throughout the experiment (Figures 1A-C). Depigmentation was the first injury caused by clomazone in all leaves treated with this herbicide (Figure 1D) irrespective of the concentration applied. This was especially apparent in areas that had a direct contact with the herbicide solution after 24 h AHA (Figure 1D). In addition, plants showed some wilting and chlorotic areas. Up to about 96 h AHA (Figure 1E) emergence of new leaves was observed, however completely depigmented. Leaf emergence completely stopped after the appearance of two or three new leaves. The plants were still turgid 96 h AHA (Figure 1E) when necrosis, mostly in the older leaves, intensified. All herbicide concentrations tested resulted in similar injuries though the effects became more severe with increasing herbicide concentrations at 96 h AHA (Table 2). Leaves exhibited partial to total necrosis at 168 h AHA (Figure 1F). By 168 h the injuries caused by the all concentrations of the clomazone herbicide led to death of *S. viridis*.

Plants treated with saflufenacil showed mostly chlorotic and necrotic points as well as some leaf wilting at 24 h AHA (Figure 1G). Plants that received the highest herbicide concentration showed severe effects at 24 h AHA (Table 2). Over time, phytotoxicity increased for all herbicide concentrations (Figures. 1G-I) where chlorosis quickly progressed to necrosis. The brownish stains of the necrosis process were seen from the lateral to the midvein of the leaf blade, followed by wilting. Plant growth stopped after the saflufenacil application for all concentrations tested. As necrosis progressed, several injuries were observed until the death of the plants (Table 2).

3.2. Pigment content

Chlorophylls *a* and *b* and carotenoids content showed no alteration in the leaves exposed 1000 g ai ha^{-1} of clomazone when compared to control plants (Figure 2A). However, when we compare treated plants at 24 and 96 h AHA there is a reduction in chlorophyll *a* and *b* content. Saflufenacil caused a reduction (near to 50%) in chlorophyll and carotenoids content at 24 h AHA (Figure 2B). At 96 h AHA samples were not collected because the plants were already severely necrotic (Figure 1H).

3.3. Anatomical, scanning and ultrastructure analyses

Anatomical, scanning and ultrastructure analyses was done to observe the effects of clomazone and saflufenacil herbicides on anatomy, surface and ultrastructure of *S. viridis* leaf blades sections. Transverse sections of *S. viridis* leaf blades exhibited Kranz architecture characteristic of C_4 plants, with a single layer of bundle sheath cells (BSC), two to three layers of mesophyll cells (MC) and two to three cells between adjacent vascular bundles (Figures 3A, B). Standard venation of Poaceae family was evidenced by the longitudinal midvein flanked by lateral veins (LV) on each side, with intermediate veins (IV) interspersed between the major veins (Figure 3A) (Junqueira *et al*., 2018). Plants showed amphistomatic leaves with stomata located in the same level of the other epidermal cells (Figure 3A). Dimensions of epidermal cells on both surfaces were variable, but the abaxial surface evidenced major discrepancy in size (Figure 3B). No chloroplasts were observed in these cells. Sclerenchyma girders were associated with the vascular bundles, especially at the midvein, and were present between the vascular bundles on both adaxial and abaxial surfaces.

The adaxial and abaxial surfaces showed single-layered epidermis, bulliform cells (Figure 3B), and unicellular (prickles) and bicellular (microhairs) trichomes. On the adaxial epidermis, above the veins (coastal region), a regular arrangement of numerous prickles in parallel rows were visible and microhairs were randomly distributed (Figures 4A, B and Figures 5A, B). In the intercoastal region hook trichomes were visible (Figure 4B). The foliar surface was covered with high density of epicuticular wax. Stomata were halter-shaped - each guard cell had a narrow, heavily thickened central portion and two bulbous ends (Figure 4C and Figure 5C).

The chloroplasts of the BSC were situated in a centrifugal position and were usually agranal or rudimentary granal (Figure 6A and Figure 7A). The MC showed numerous chloroplasts randomly distributed toward the cell wall, with fusiform shape, containing well-arranged thylakoid membranes, compact grana stacks and plastoglobules (Figures 6B, C and Figures 7B, C). Chloroplasts of MC of plants that had not received herbicides had prominent starch grains and mitochondria in close proximity (Figure 6B). BSC chloroplasts appeared to be bigger than MC chloroplasts (Figures 6A, B and Figures 7A, B).

3.3.1. Clomazone

Leaves of *S. viridis* exposed to clomazone showed changes in their surface, anatomy, and ultrastructure. The anatomical analysis of leaf blade cross sections and surface analysis

with SEM after 24 h of clomazone showed two different architectures (Figure 3C, Figure 4D and Figure 8A); necrotic areas, evidenced by direct contact with the clomazone application (Figures 4D, E), and unaffected areas with similar structure to the control plants (Figures 4H, I and). In the necrotic areas, collapsed tissue was accompanied by marked changes in epidermal cells (Figure 3C and Figure 4E). In necrotic areas, after 24 h AHA, leaf blade thickness drastically decreased, mesophyll and bundle sheath cells were collapsed and no intercellular spaces were observed (Figure. 3C and Figure 8A). Altered epidermal cells featured loss of epicuticular wax and apparent cell wall degradation (Figure 4E). Furthermore, in the necrotic region the size of stomata and pores was affected (Figure 4F and Figures 8B, C). Morphology of guard cells and subsidiary cells was altered and the density of epicuticular wax was apparently reduced (Figure 4F). Silicified long cell between two prickles were more obvious after clomazone application in the epidermal cells in the coastal region (Figures 4G). Cell wall thickness of adaxial epidermal cell decreased in relation to control plants (Figure 6D) and MC chloroplasts showed spherical shape, rupture of envelop membrane and disintegration of thylakoids, and grana stacking (Figures 6E, F). Additionally, BSC showed loss of thylakoid integrity and starch grains scattered in the cytoplasm. Unaffected areas have organized mesophyll and bundle sheath cells and a well preserved morphology (Figure 3C). The proportion of cell size in the different tissues i.e. epidermis and mesophyll remained unchanged (Figure 8A). Surface (Figures 4H, I) and cell ultrastructure were similar to untreated plants, showing MC chloroplast with well-arranged thylakoids (Figures 6G-I).

After 96 h AHA clomazone caused cellular compression bringing the coastal regions closer together, especially in necrotic areas (Figure 5D) and affected the shape of trichomes (Figures 5E, G). Stomata showed the same alterations, guard cell and subsidiary cells shrunk, and the epicuticular wax showed an apparent reduction too (Figure 5F). The irreversible damage includes total disruption of chloroplasts in the BSC and MC (Figure 7E) as well as the decrease in the thickness of cell wall in the adaxial epidermis (Figure 7D, 96 h AHA). Unaffected areas of leaves treated with clomazone revealed no alteration on the surface and stomata shape (Figures 5H, I), and absence of starch grains in the BSC chloroplasts, similar to the control plants (Figures 7A, F), although swelling of thylakoids membranes was observed (Figure 7F). Some MC chloroplasts showed accentuated grana stacking, increased intra thylakoid space and the emergence of peripheral reticulum (Figures 7G-I).

3.3.2. Saflufenacil

Setaria viridis leaves exposed to saflufenacil showed drastic changes in anatomy,

surface and ultrastructure. The micromorphology and organization of epidermal and bulliform cells, MC and BSC at 24 h AHA were altered (Figure 3E) evidenced by the reduction in leaf thickness (Figure 8A). Unlike the effect observed for clomazone herbicide, saflufenacil induced alterations in the whole leaf blade evidenced by reduction in leaf thickness, extensive compression of MC and flattening of epidermal cells, although the lumens of BSC were preserved in some areas (Figure 3E and Figure 8A). SEM analyses showed alterations in the epidermal cells of the intercoastal zone (Figure 4J): flattening of the epidermal cells and loss of the parallel arrangement, including possible partial degradation of cell wall (Figure 4K), similar to clomazone effect (Figure 4E). Stomata exhibited an elongated shape with a larger pore when compared to the control plants (Figure 4L and Figure 8C), but with smaller area (Figure 8B). Guard and subsidiary cells were shrunk and the density of epicuticular wax was reduced (Figure 4L). The ultrastructure of BS and MC chloroplast was severely affected (Figures 6K, L). Thylakoid membranes were highly disorganized and the amount of starch grain in BSC chloroplast appeared to be higher than in the control plants (Figures 6A, K). MC chloroplast showed disruption of the envelope (Figure 6L). After 96 h, the alterations on the leaf surface were also very similar to those caused by clomazone (Figure 3F and Figures 5J-L), showing decrease of epidermal cells thickness (Figure 7J). MC chloroplasts showed strong alterations evidenced by increase in the number of plastoglobule and disorganization of thylakoids (Figures 7K, L).

4. Discussion

General observations

Leaf injuries were observed for all concentrations of the herbicides that were tested demonstrating the sensitivity of *Setaria viridis* to clomazone and saflafenacil, even under the lowest concentrations of herbicide. For both herbicides, at 168 h AHA some plants that have been treated with the smallest concentration were still alive, although partially necrotic and smaller in size.

It is important to consider that the effect attributed to clomazone and saflufenacil are likely to be a synergetic effect of the other substances present in the commercial formula of both herbicides, such as surfactants used to aid the penetration of the active (Malkomes, 2000). The presence of epicuticular wax can reduce the uptake of herbicides by plants has been reported to provide a barrier to herbicide uptake to *S. viridis* (Carr *et al*., 1986). Here, the contact of clomazone and saflufenacil on the surface of *S. viridis* leaf blades affected the

epicuticular wax deposition, mainly in stoma areas (Figure 4L and Figure 5L). The lack of epicuticular wax was also reported in stomata cells of *Triticum aestivum* L. (Yilmaz and Dane, 2012) and according to literature, the uptake of herbicides occurs mainly through these cells (Franke, 1969; Kerstiens, 1996). Moreover, the hydrophilic nature of herbicides can cause alterations in the density of the epicuticular wax (Kostakis *et al*., 2013; Alves *et al.*, 2014). The structure and arrangement of the epicuticular wax also influence the absorption of the herbicide in plants (Santier and Chamel, 1998). *Setaria viridis* exhibit stomata with a crystalline wax structures formed by flat crystalloids perpendicularly oriented and grouped in rosettes (Figure 5C and 6C). Although stomata constitute an easy entrance for volatile herbicides, when in aqueous solution this pathway is not at disposal due to the high surface tension of the spray solution (DiTomaso, 1999). Moreover, herbicides have a low uptake via stomata due to their lower number on the adaxial surfaces of most species with amphistomatic leaves and it is challenging for the droplets to reach the abaxial surface in agricultural spraying (Alves *et al*., 2014). Lower number of stomata on the adaxial surface was confirmed for *S. viridis* (Sanyal *et al*., 2006) and here, also it was observed a reduction on its dimensions to both herbicides (Figure 8B and C). Besides this, the fact that stomata were closed during various times of the day implies that this type of absorption is of minor importance (Ferreira *et al*. 2002). However, breaking the surface tension of a spray solution on the leaves through use of silicone surfactants can promote greater spreading of the product and allows the stomata to play an important role in the permeation of herbicides (Alves *et al*., 2014).

Clomazone effects

It was possible to observe the typical effects of clomazone in *S. viridis* leaves, characterized by complete depigmentation that emerged after the herbicide had been applied (Figure 1E). This is associated with the action of clomazone as an inhibitor of carotenoids biosynthesis followed by degradation of chlorophyll caused by photo-inhibition (Kaňa *et al.*, 2004). Carotenoids are pigments involved in photosynthesis, photoprotection and membrane stabilization (Vítek *et al*., 2017; Biswal, 1995). For example, xanthophyll carotenoids have an important role of dissipation of excess excitation energy through the xanthophyll cycle, considered a key photoprotective mechanism in higher plants (Adams and Adams, 1992; Lunch *et al.*, 2013). Clomazone did not affect the pigment content when compared to control, however, there was a decrease, mostly in the chlorophyll *a* content, when we compared the two sample times (24 and 96 h) (Figure 2A). This difference could be related to the increase

of necrosis points over time, mainly due to the herbicide's direct contact on the *S. viridis* leaves. A similar reduction in the content of photosynthetic pigments as an effect of the clomazone application was observed in *Hordeum vulgare* L. (Kaňa *et al.*, 2004) and this preferential reduction of chlorophyll *a* overtime suggested degradation of the chlorophyll *a*-containing light-harvesting complexes (LHCs) in thylakoid membranes. Similar decreases were observed under higher concentrations of clomazone in plantlets leaves of tobacco (Darwish *et al.*, 2014), in adult leaves of tobacco and tomato (Darwish *et al.*, 2013; Scott *et al.*, 1994), and in the adult leaves of barley (Kaňa *et al.*, 2004). Here, therefore, after the pigment content of *S. viridis* in the young leaf blades was drastically affected by clomazone, the pigment pre-existing in older leaf blades may have suffered degradation by photo-inhibition.

Anatomically, epidermis, including bulliform cells, and the mesophyll were the most affected tissues. The reduced number of epidermal cells, mesophyll and bundle sheath can lead to inhibition of photosynthesis in plants treated with the clomazone due to disturbance in gas exchange (Harris *et al.*, 1986). It was clear that clomazone induced dehydration stress that led to histological and cellular disorganization. These symptoms were similar to those observed for saflufenacil. The reduction in mesophyll thickness (Figure 8A) may be relate to loss of cellular spaces as reported to arborescent species treated with clomazone (Cabral *et al.*, 2017). The decrease of tissue integrity can be related to a loss in stability of membrane proteins (Cabral *et al.*, 2017). Necrotic areas containing tissues under a fast collapse showing cell content disruption (Figures 3C, D and Figures 4D-F and Figures 5D-G) could be related to "non-target site resistance" mechanisms associated with reduced cell uptake capacity and/or translocation (Cardoso-Gustavson *et al.*, 2018). Fast cell collapse is very similar to hypersensitive response to a pathogen infection (Cardoso-Gustavson *et al.*, 2018), with a localized defense reaction feature involving the induction of specific structures in discrete groups of quickly dying cells (Vollenweider *et al.*, 2003) acting to the reduce translocation of herbicide by fast collapsing the uptake cells (Cardoso-Gustavson *et al.*, 2018). Cell collapse can be seen as necrosis points (Figure 1E and Figure 8A) accompanied by changes in cell ultrastructure such as: the decrease in cell wall thickness in the adaxial epidermis (Figure 6D and Figure 7D); chloroplasts in MC with a spherical shape, rupture of the envelop membrane, disintegration of thylakoids and grana stacking (Figures 6E, F), loss of thylakoid integrity, and starch grains scattered in the cytoplasm of BSC. Similarly, ultrastructural changes were observed in the necrotic tissues of *Matricaria inodora* and *Viola arvensis* treated with

hydroxybenzonitrile herbicide (Sanders and Pallett, 1986). Inhibition of chloroplast development along with reduction in grana formation and chlorophyll content at 24 h AHA (Figures 6E, F) were also reported in pea seedlings under the influence of 2.2'-bipyridyl (Mostowska and Siedlecka, 1995) and in maize treated with 1.10-pheanthroline (Mostowska, 1998).

Clomazone induced the emergence of peripheral reticulum and a swelling in the thylakoids along with intense grana stacking in MC chloroplasts at 96 h AHA (Figures 7G-I). The function of the peripheral reticulum is not fully understood, although its development has been observed in several plants species (Gracen *et al*., 1972; Szczepanik and Sowinski, 2014). It seems to be associated with the transport of metabolites between MC and BSC, aiding to the process of photosynthesis (Szczepanik and Sowinski, 2014) or with photorespiration rate (Hilliard and West, 1971). Some reports have shown an increase in the peripheral reticulum in plants exposed to high light intensities and water deficit (Black and Mollenhauer, 1971; Grace e*t al*., 1972; Utrillas and Alegre, 1997). Our research group has observed that when *S. viridis* is exposed to heat stress there is an increase of peripheral reticulum in MC chloroplasts (unpublished data). The swelling of the thylakoids can be attributed to membrane depolarization and changes in permeability, as reported for maize seedlings due to a chelator effect of 1.10-phenanthroline (Mostowska and Siedlecka, 1995). Intense grana stacking can be interpreted as an attempt to compensate for the intense destruction in necrotic areas and a result of higher photosystem II activity in preserved structures and areas not directly impacted by the herbicide. Saline stress also caused grana stacking in maize, however, in the chloroplasts of BSC instead in the chloroplasts of the MC, after a complete destruction of the MC (Hasan et al. 2006).

Saflufenacil effects

Here, the commercial formulation of saflufenacil caused loss of wax and reduction of cell wall thickness of epidermal cells (Figures 3E, F and Figures 4J-L and Figures 5J-L), which could facilitate the penetration of the herbicide. The symptoms observed in *S. viridis* due to saflufenacil application were expected considering the mechanism of action described for this herbicide. Inhibition of the PPO enzyme causes protoporphyrinogen accumulation that spreads to the reaction centers leading to a process of oxidation through its interaction with oxygen radicals. This process leads to the production of excessive reactive oxygen species (ROS) in the presence of light and absence of protective molecules, causing loss of membrane

integrity, leading to ion leakage and water loss (Grossman *et al.*, 2010; Oliveira Junior, 2011, Langaro *et al.,* 2017). Thus, the herbicide mechanism of action is not directly associated with interruption of the electron transport chain itself, but with a cascade of events that lead to photosynthesis inhibition. Necrotic tissue (Figure 1G) observed at 24 h AHA, evidenced the above damage, initiated by saflufenacil application as reported for *S. italica* (L.) P. Beauv (Reddy *et al.*, 2014).

White spots along leaf blades were observed in some leaves immediately following saflufenacil application (Figure 1H). Initially, plants were wilted with points of necrosis that evolved to necrosis of the whole leaf tissue. This response was faster in the highest concentration (Table 2). This dose-dependent level of injury over time was also reported for rice (Camargo *et al.*, 2012) and broadleaf weeds (Geier *et al.*, 2009).

Inhibition of PPO activity in *S. viridis* caused by the saflufenacil application also affected degradation at the cellular level, including disruption and breakdown of thylakoid membranes and appearance of a large number of plastoglobules. The cell damage in MC and BSC could be a result of excessive production of ROS leading to lipid peroxidation among other detrimental effects (Langaro *et al*., 2017). Destruction of chloroplasts associated with ROS production was also reported in maize (Hu *et al*., 2006). Increases in the appearance of plastoglobules in chloroplast can be associated with lipid peroxidation too, as observed in plants during the senescence process (Liu, 2016). Similarly, differences in quantity and extent of plastoglobules as well as peroxide accumulation were observed in plants under stressed conditions such as nitrogen starvation, drought, temperature variations and exposure to excessive light (Halliwell and Gutteridge, 2015; Rottet *et al*., 2015). Here, plants treated with saflufenacil also exhibited a higher level of starch grains accumulation than control plants. This could be attributed to decreases in the leaf tissue ability to load sucrose into the phloem (Figure 6K), as happened with mung bean plants treated with Metsulfuron (Kaushik, 2006).

5. Conclusions

- Genome size, plant size and life-cycle of *S. viridis* proved more than adequate for evaluating the effects of herbicides on leaf blade structure and ultrastructure.
- The effects of clomazone and saflufenacil have been clearly shown and described and will serve as a reference to those studying C_4 crop plants and herbicides.
- Clomazone caused an “albino” appearance in young leaves and necrotic

areas by herbicide contact.

- Saflufenacil caused injuries to foliar tissues, such as necrosis and depigmentation and deformation in chloroplasts of Bundle sheath and Mesophyll.
- Not unlike the most famous plant model, *Arabidopsis thaliana* (L.) Heynh, that through research has improved and accelerated studies of plant biology, one hopes that knowledge gained by studying *S. viridis* will have many indirect positive impacts on global food security and herbicide management.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

This work is part of the doctoral thesis of VALENCA, D.C. in Plant Biotechnology (Programa de Pós-Graduação em Biotecnologia Vegetal e Bioprocessos, Universidade Federal do Rio de Janeiro, Brazil), supported by a scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) (141802 / 2016-2). The authors wish to thank to the Weed and Pesticides in the Environment (PDPA-UFRRJ) research group by partnership and Professor Ricardo Vieira for support, comments and suggestions that contributed to the paper.

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Tables

Table 1. Herbicide treatments and their respective concentration of the commercial dose applied over *Setaria viridis*.

Common name	Trade name	Herbicide composition	Concentrations (g ai ha^{-1})			
		(g ai L^{-1})	0.5D	D	1.5D	2D
		Clomazone - Carotenoid biosynthesis inhibitor				
Clomazone	Gamit	500	500	1000	1500	2000
		Saflufenacil - PPO inhibitor*				
Saflufenacil	Heat®	-	49	98	147	196

2. D=commercial dose; ai= active ingrediente; *PPO: protoporphyrinogen IX oxidase.

Table 2. *Setaria viridis* visible injuries as a response to clomazone and saflufenacil herbicides applications.

Herbicide	Concentration (g ai ha^{-1})	Visible Injury (%)		
		Time after application		
		24h	96h	168h
Clomazone - Carotenoid biosynthesis inhibitor				
Clomazone	500	L[1,2,3]	M[1,2,3,4]	S[1,2,3,4,5]
	1000	L[1,2,3]	M[1,2,3,4]	S[1,2,3,4,5]
	1500	L[1,2,3]	S[1,2,3,4]	S[1,2,3,4,5]
	2000	L[1,2,3]	S[1,2,3,4]	S[1,2,3,4,5]
Saflufenacil – PPO inhibitor*				
Saflufenacil	49	M[1,2,4]	S[5]	S[5]
	98	M[1,2,4]	S[5]	S[5]
	147	S[1,2,4]	S[5]	S[5]
	196	S[1,2,4]	S[5]	S[5]

Injury was estimated visually using the following scale: Light (L) 0 – 33%; Medium (M) 34 – 66%; Severe (S) 67 – 100%. Percentage of injury according with Frans (1986); (1) leaf tissue exhibiting chlorotic points of herbicide contact; (2) wilting on the leaf tissue; (3) depigmentation of leaves; (4) leaf tissue exhibiting necrosis points of herbicide contact; (5) complete leaf tissue necrosis. *PPO: protoporphyrinogen IX oxidase.

Figures

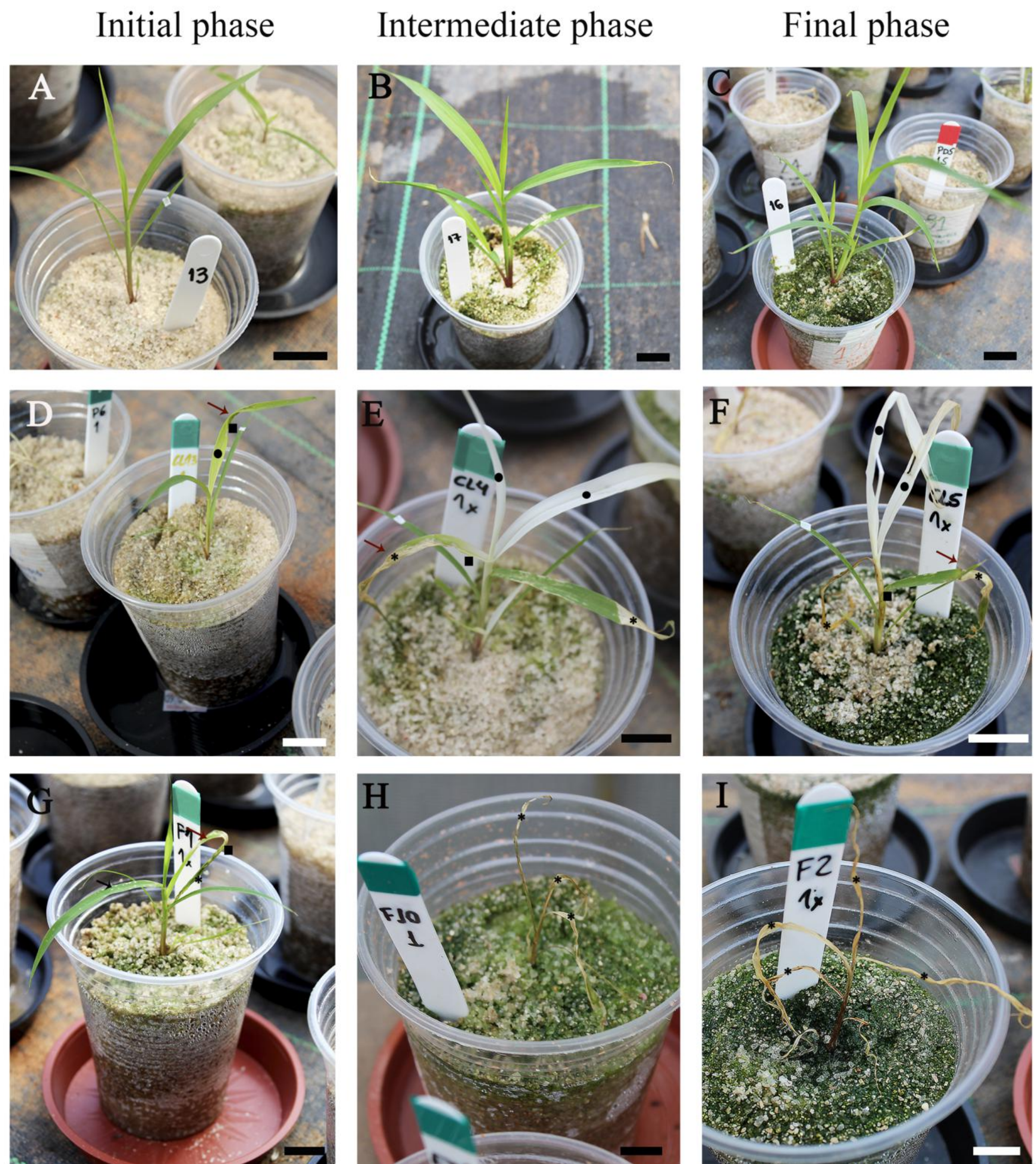

Fig. 1. Images of *Setaria viridis*. A-C, control plants; D-F, clomazone-treated plants and G-I, saflufenacil-treated plants at 24, 96 and 168 h after herbicide application (AHA), respectively. All images of herbicide-treated plants were performed using commercial dosages. Photographs by SILVA B O and VALENCA D C. Scale bars: 10 mm. Symbols represent different visible injuries: chlorosis (squares filled); necrosis (asterisks); depigmentation (circles filled); wilting (red arrows); and depigmentation points (black arrows).

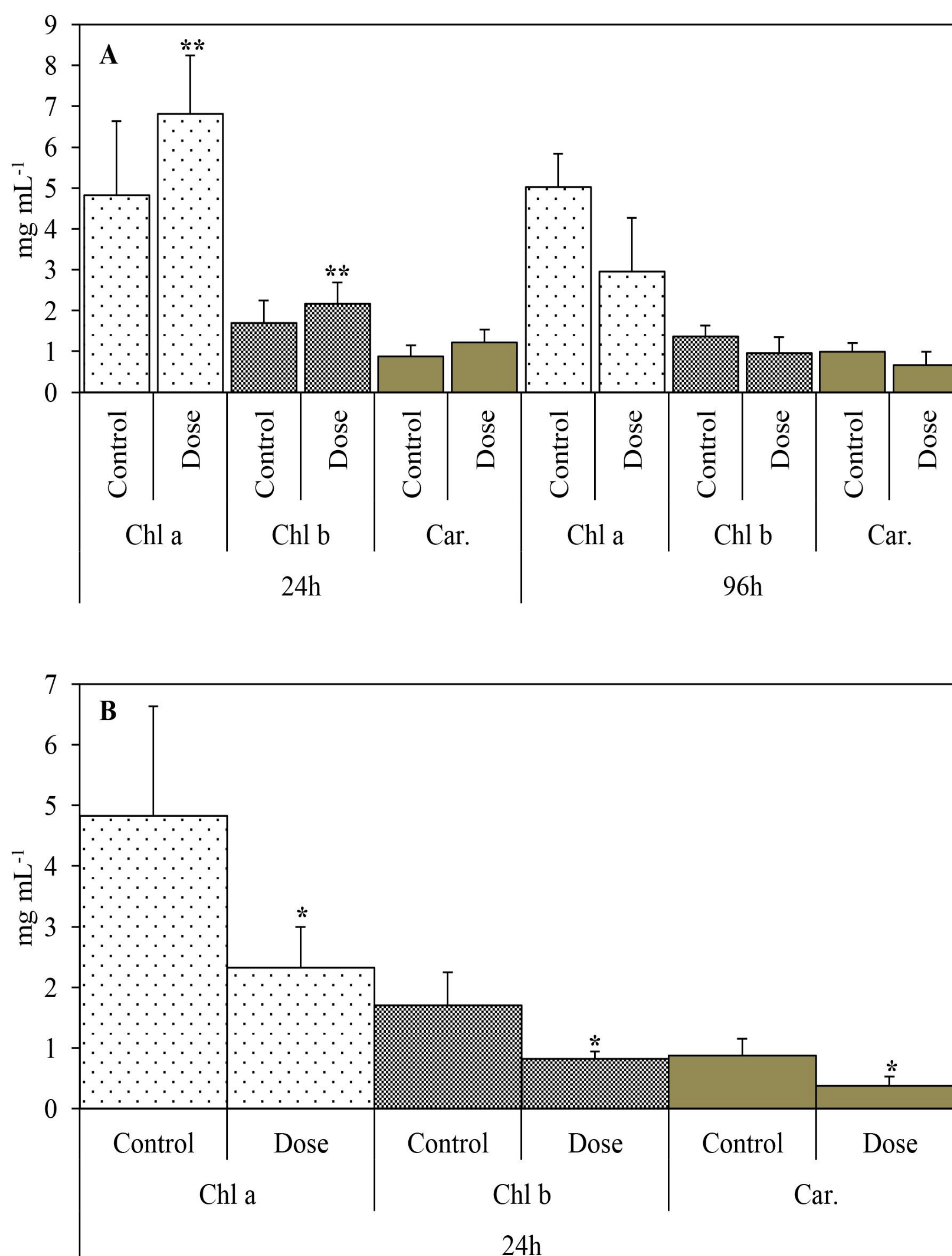

Fig. 2. Chlorophyll and carotenoid concentration per leaf area of *Setaria viridis* treated with A, clomazone and B, saflufenacil. Chlorophyll *a* (Chl *a*); Chlorophyll b (Chl *b*); and Carotenoids (Car.). Bars represent mean ±SE (n=5). Asterisk on the bars indicate significant differences compared with the control level by Tukey-Kramer HSD (P≤0.05), whereas a double asterisk indicates differences between exposure time using the Tukey-Kramer HSD (P≤0.05) in a factorial scheme considering herbicide exposure times and herbicide application dose. The control represents plants that did not receive herbicide application and Dose represents plants that received the commercial dose of each herbicide (1000 and 98 g a.i ha^{-1} of clomazone and saflufenacil respectively).

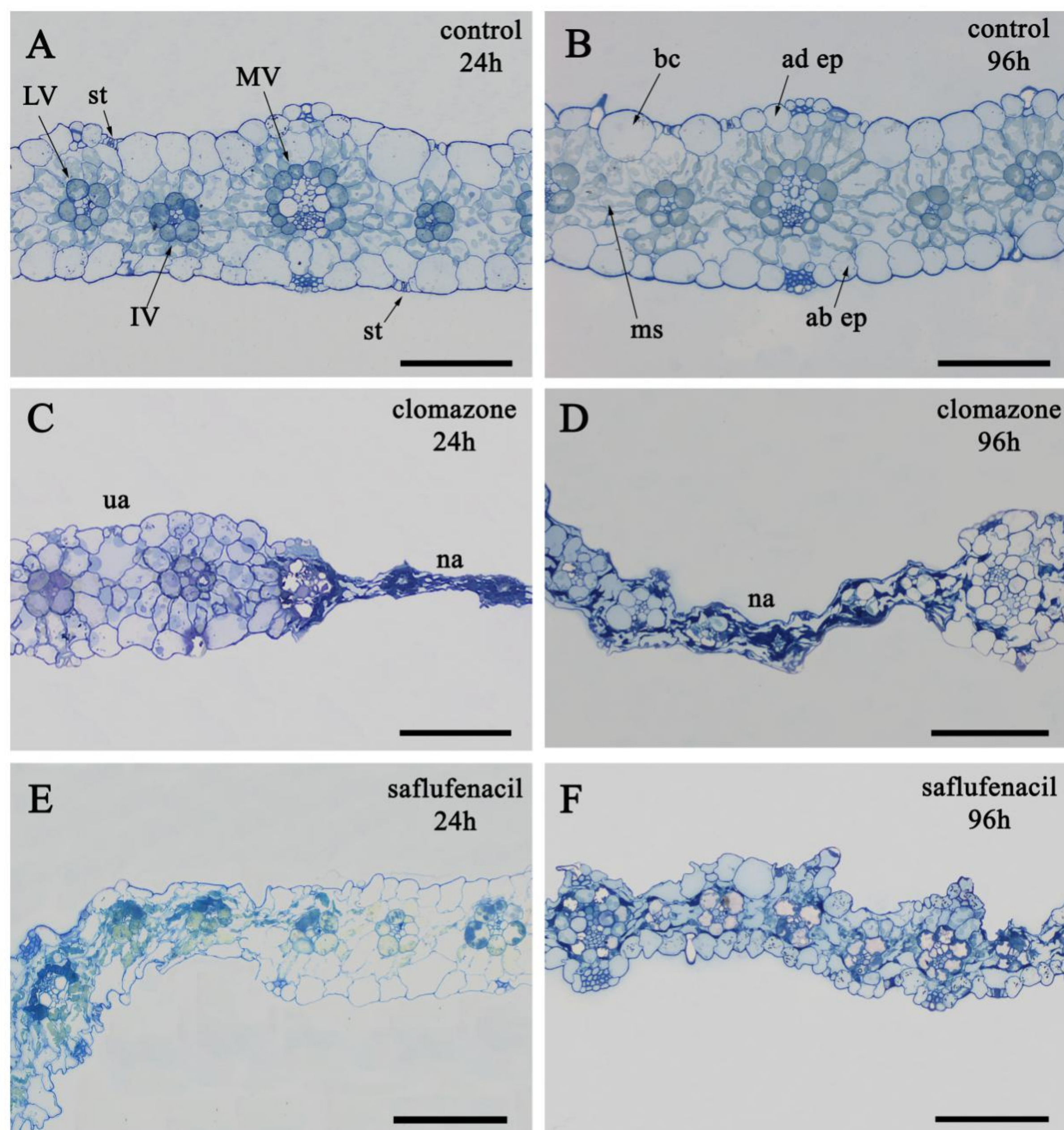

Fig. 3. Cross section of *Setaria viridis* leaf blade. Control plants at A, 24 h after herbicide application (AHA) and B, 96 h AHA. Clomazone-treated plants at C, 24 h AHA and D, 96 h AHA. Saflufenacil-treated plants at E, 24 h AHA and F, 96 h AHA. All images of herbicide-treated plants are with the commercial herbicide dose: 1000 and 98 g a.i ha^{-1} of clomazone and saflufenacil respectively. Scale bars: 100 µm. Midvein (MV); lateral veins (LV); intermediate veins (IV); epidermis adaxial (ad ep); epidermis abaxial (ab ep); mesophyll cells (m); buliforms cells (bc); stoma (st), unaffected area (ua); and necrotic area (na).

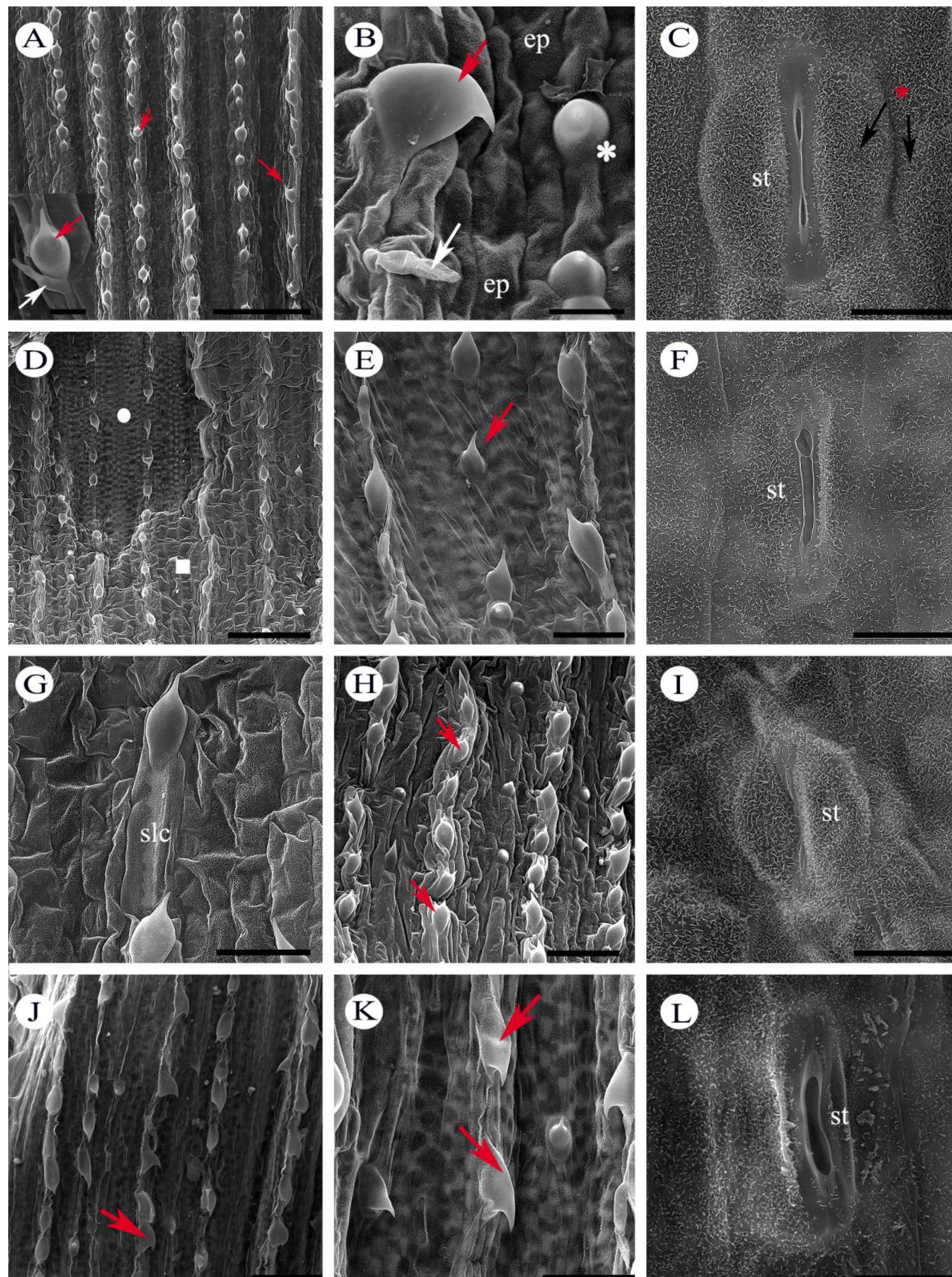

Fig. 4. Scanning Electron Microscopy of adaxial surfaces of *Setaria viridis* leaf blade at 24 h after herbicide application. A-C, Control plants showing epidermis cells, unicellular and bi-cellular trichomes and stomata; D-G, Clomazone-treated plants (necrotic area); H-I, Clomazone-treated plants (unaffected area); J-L, Saflufenacil-treated plants. All images of herbicide-treated plants are with the commercial herbicide dose: 1000 and 98 g a.i ha^{-1} of clomazone and saflufenacil respectively. Scale bars: 10 µm (C, F, I, L); 20 µm (A-detail, B), 50 µm (E, G, K); 100 µm (H, J); 200 µm (A, D). Trichomes prickles (red arrow); bicellular microhairs (white arrow); hook trichome (white asterisks) stomata (st); epidermal cell (ep); epicuticular wax (red star); necrotic area (white circle); preserved area (white square); and silicified long cell (slc).

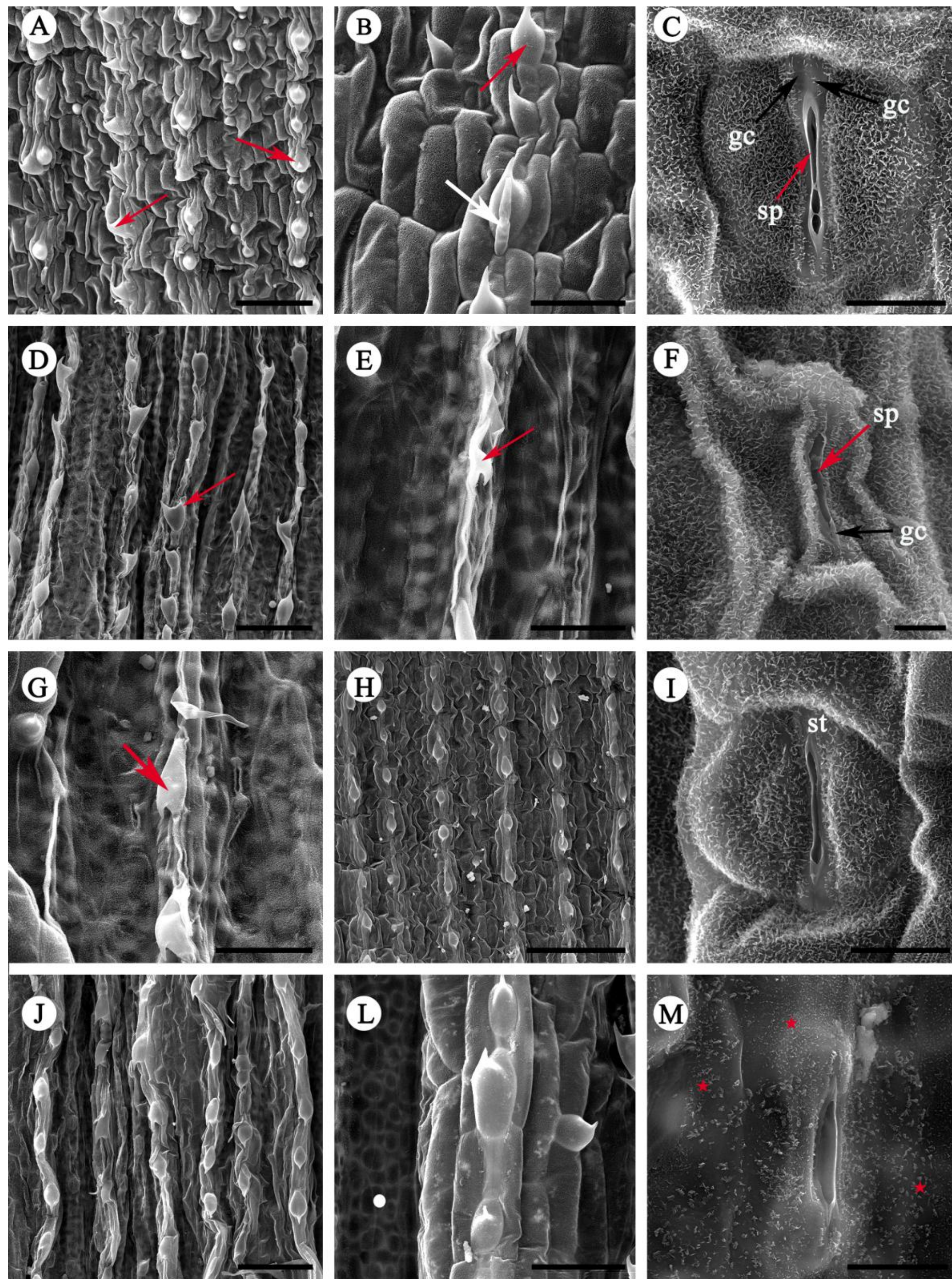

Fig. 5. Scanning Electron Microscopy of adaxial surfaces of *Setaria viridis* leaf blade at 96 h after herbicide application. A-C, Control plants showing epidermis cells, unicellular and bi-cellular trichomes and stomata; D-G, Clomazone-treated plants (necrotic area); H-I, Clomazone-treated plants (unaffected area); J-L, Saflufenacil-treated plants. All images of herbicide-treated plants are with the commercial herbicide dose: 1000 and 98 g a.i ha^{-1} of clomazone and saflufenacil respectively. Scale bars: 5 µm (F); 10 µm (C, I, L); 50 µm (B, E, G, K); 100 µm (A, D, J); 200 µm (H). Prickles (unicellular trichomes; red arrow); bicellular microhairs (white arrow); stomata (st); stomatal pore (sp); guard cell (gc); detail of cellular lumen (white circle); epicuticular wax reduction (red star).

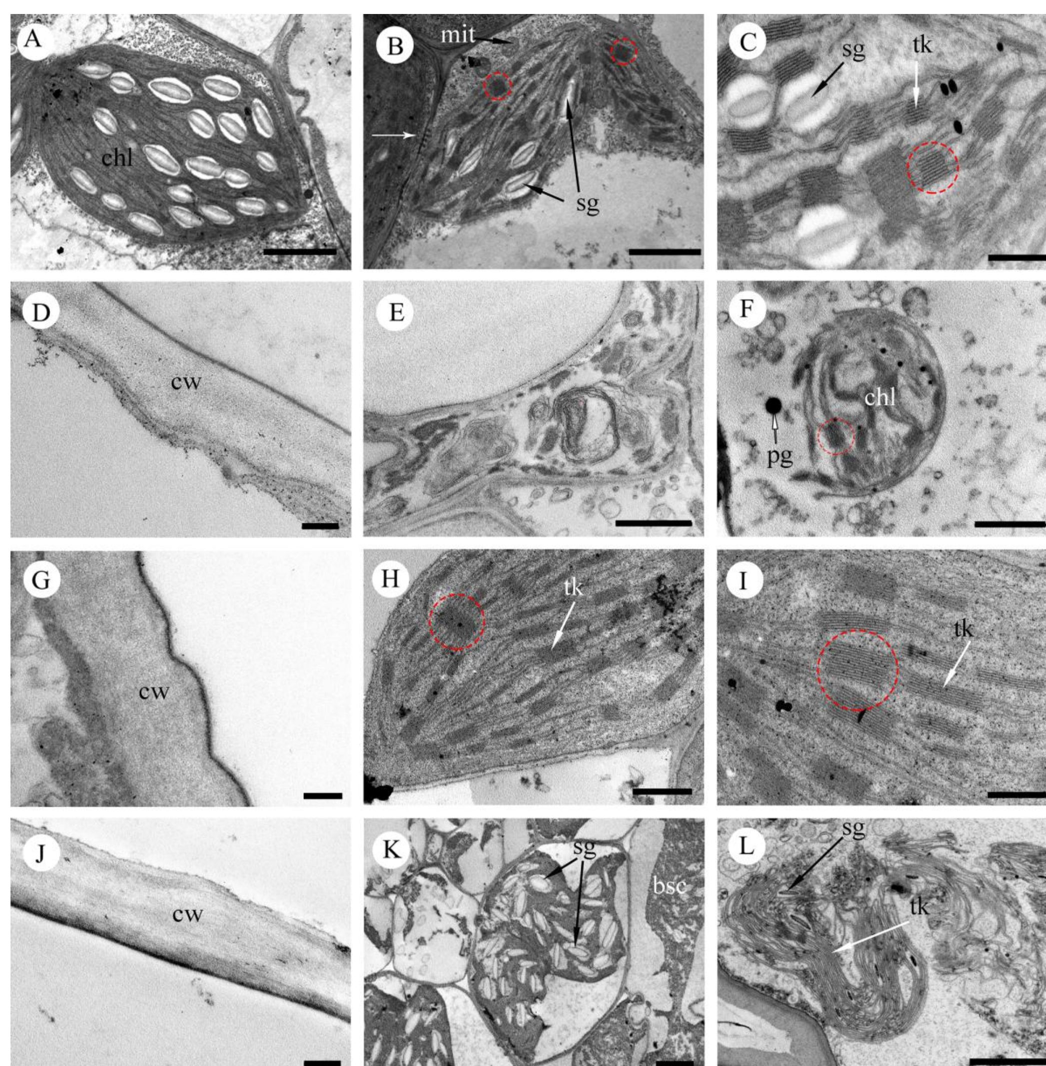

Fig. 6. Transmission Electron Microscopy of *Setaria viridis* leaf blade from the control and herbicide-treated plants at 24 h after herbicide application. A, Bundle sheath (BSC) chloroplast with rudimentary grana and a high amount of starch grain; and B, C, Mesophyll (MC) chloroplast with fusiform shape, containing well-arranged thylakoid membranes, grana stacks, little starch grain and plastoglobules in control plants. D, Cell wall thickness of the adaxial epidermis; and E, F, MC chloroplasts with spherical shape, rupture of envelop membrane and disintegration of thylakoids and grana stacking in clomazone-treated plants (necrotic area). G, Cell wall thickness in the adaxial epidermis (this thickness is the same of control that not shown); and H, I, MC chloroplast with the same appearance of control in clomazone-treated plants (unaffected area). J, Cell wall thickness of the adaxial epidermis; K, BSC chloroplasts with the amount of starch grain appeared higher than control; and L, MC chloroplast with a high disorganization in thylakoid membranes and envelope membrane rupture. All images of herbicide-treated plants are with the commercial herbicide dose: 1000 and 98 g a.i ha^{-1} of clomazone and saflufenacil respectively. Scale bars: 0.2 µm (D, G, J); 0.5 µm (C, I); 1.0 µm (E, H); 2.0 µm (A, B, F, K, L). Chloroplast (chl); mitochondria (mit); granum (dashed red line); starch grains (sg); thylakoids membranes (tk); cell wall (cw); plastoglobuli (pg); plasmodesmata (white arrow); bundle sheath cell (bsc).

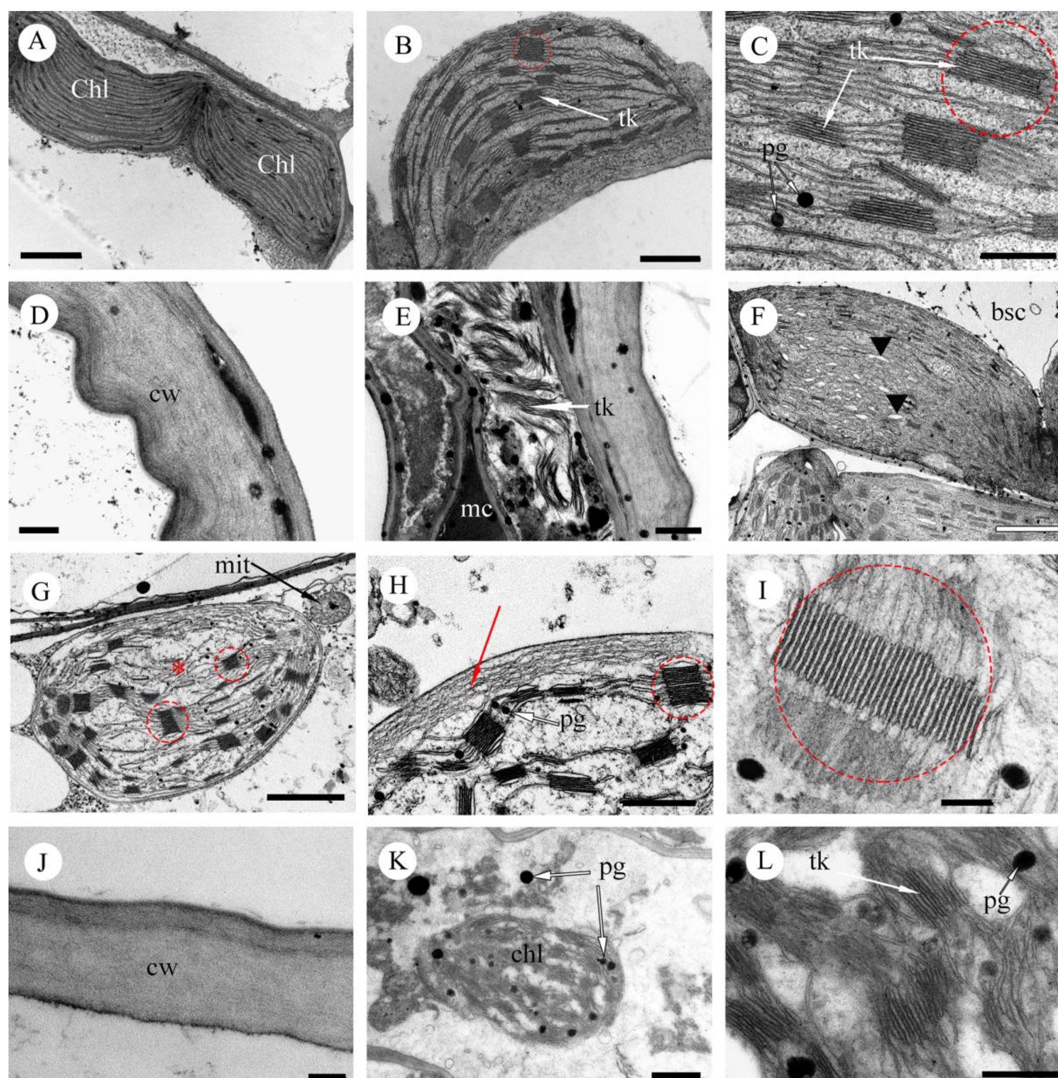

Fig. 7. Transmission Electron Microscopy of *Setaria viridis* leaf blade from control and herbicide-treated plants at 96 h after herbicide application. A, Bundle sheath (BSC) chloroplast with absence grana; and B, C, Mesophyll (MC) chloroplast with fusiform shape, containing well-arranged thylakoid membranes, grana stacks, and little plastoglobules in control plants. D, Cell wall thickness in the adaxial epidermis; and E, MC cells shown completely rupture of envelope membranes in clomazone-treated plants (necrotic area). F, BSC chloroplast with absence of starch grains similar to the control and swelling of thylakoids membranes; and G-I, MC chloroplasts with accentuated grana stacking, intrathylakoid space and emergence of peripheral reticulum in clomazone-treated plants (unaffected area). J, Cell wall thickness of the adaxial epidermis; and K, L, MC chloroplast with a high disorganization in thylakoid membranes. All images of herbicide-treated plants are with the commercial herbicide dose: 1000 and 98 g a.i ha^{-1} of clomazone and saflufenacil respectively. Scale bars: 0.2 µm (D, F, H, I, J); 0.5 µm (C, E, L); 1.0 µm (B, K); 2.0 µm (A, G). Chloroplast (chl); granum (dashed red line); thylakoids membranes (tk); plastoblobules (pg); cell wall (cw); mesophyll cell (mc); swelling of thylakoid membrane (black triangle); distorted thylakoids (red asterisks); mitochondria (mit); peripheral reticulum (red arrow).

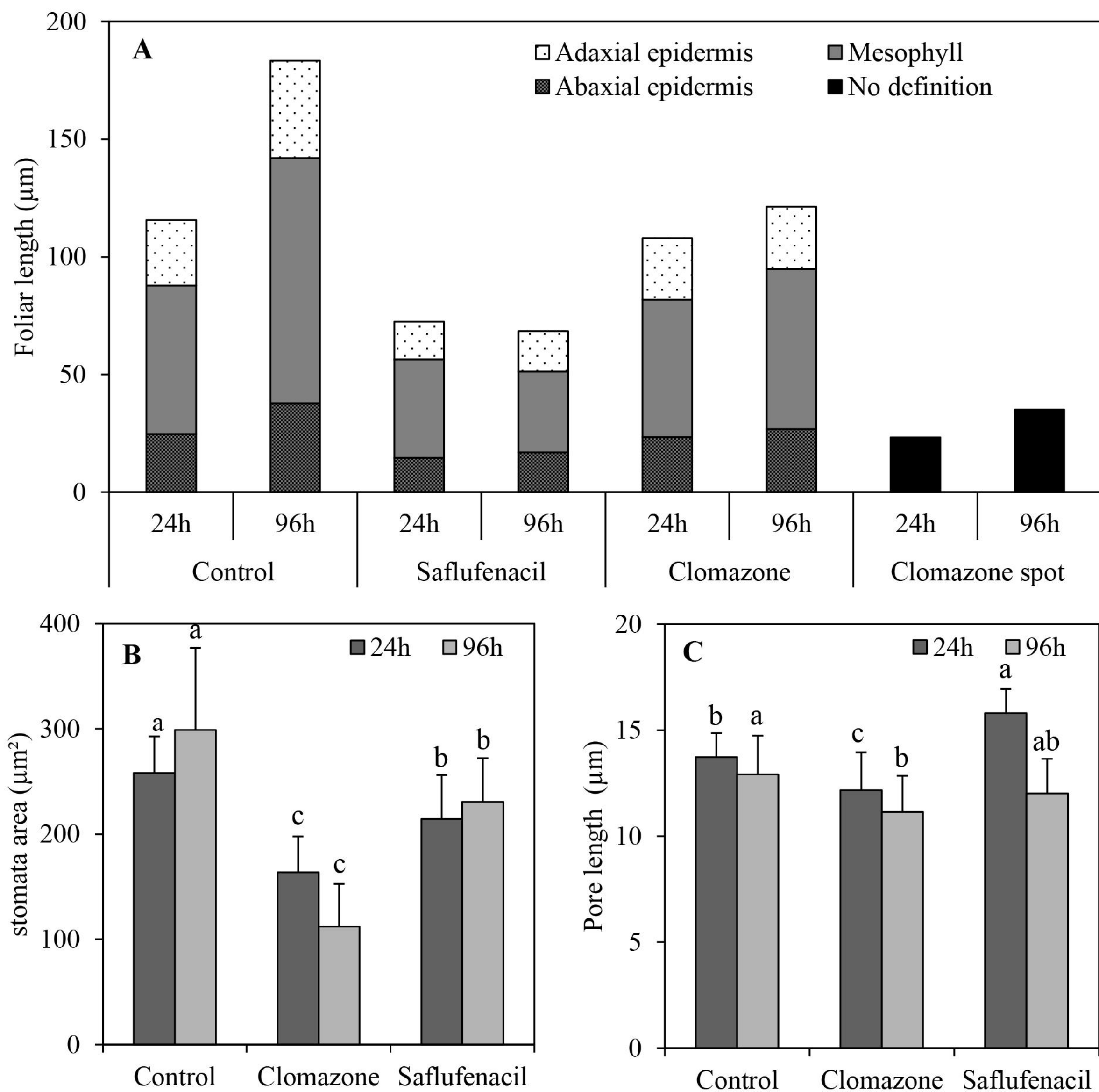

Fig. 8. Morphometric analysis of *Setaria viridis* leaf blade from different treatments at A, 24 and 96 h after herbicides applications (AHA). B, Stomata area of plants from different treatments. C, Pore length of plants from different treatments. All images of herbicide-treated plants are with the commercial herbicide dose: 1000 and 98 g a.i ha-1 of clomazone and saflufenacil respectively Bars represent mean ±SE (n=20).

5. CAPÍTULO II – ARTIGO II

Physiological and molecular responses of *Setaria viridis* to osmotic stress

Artigo publicado no periódico Plant Physiology Biochemistry (ISSN 0981-9428) sob o número de DOI: 10.1016/j.plaphy.2020.07.019. (ANEXO II)

Physiological and molecular responses of *Setaria viridis* to osmotic stress

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ABSTRACT

Drought-tolerant species, such as *Setaria viridis*, a C_4 model plant, make physiological and biochemical adjustments water limitation and recover from the stress upon its release. We investigated *S. viridis* (A10.1 accession) responses to continuing osmotic stress. The osmotic stress was imposed using polyethylene glycol (PEG) 8000 (7.5%) for 10 days. Morphological traits and stomatal conductance were measured daily for the 10 days. On days 6 and 10, the following traits were measured separately for root and shoot: relative water content (RWC), osmotic potential (OP), electrolytic leakage (EL), and proline content. qPCR analysis was used to evaluate the expression of five selected genes in roots (*SvLEA*, *SvDREB1C*, *SvPIP2-1*, *SvHSP20,* and *SvP5CS2*), and chlorophyll *a* fluorescence was measured on three key days. The morphological

data demonstrated a drastic reduction in shoot biomass as an effect of water deficit caused by the osmotic stress. Shoot biomass reduction could be associated with putative ABA-dependent signaling involved in *SvDREB1C* expression. Stomatal conductance and photosynthesis were severely affected up until day 6, however, stomatal conductance and some photosynthetic parameters such as F_V/F_M, ABS/RC, and DI_0/RC showed total or slight recovery on day 10. Root EL decreased in treated plants suggesting an investment in membrane protection by osmoregulator expression such as dehydrin (*SvLEA*) and proline (*SvP5CS2*) genes. Our data suggest that *S. viridis* exhibited a partial recovery from an imposed and constant osmotic stress within 10 days.

Key-words: C_4 model plant; gene expression analyses; morphological traits; photosynthesis; proline accumulation; water deficit.

1. Introduction

Drought events associated with global climate change increasingly cause severe losses in agriculture, threatening food security worldwide (Salgado *et al*., 2020). In this scenario, our knowledge about crops with high drought tolerance is key for the development of tools to manage cultivated plants under water restriction. The complex plant mechanisms to survive under limiting environments are key to define an approach for the challenges agriculture will face in the near future (Salgado *et al*., 2020). Plant drought tolerance is related to the ability to maintain metabolism while several interactions at physiological and molecular levels occur (Cia *et al*., 2012).

Setaria italica is a C_4 Poaceae found in a variety of environments as it has mechanisms that enable water use efficiency (WUE). While maize and wheat require 470 and 510 g of water to accumulate 1 g of dry biomass, respectively, *S. italica* requires only 257 g (Diao, 2005; Diao, 2007). *Setaria viridis* (L.) P. Beauv., a wild ancestor of *S. italica*, is a model plant with a C_4-NADP-ME subtype of photosynthesis mechanism (Brutnell *et al*., 2010). The CO_2 fixation of this photosynthetic mechanism has an intrinsic relationship with improved water and nitrogen use efficiency (Edwards *et al*., 2010). *S. viridis* is found worldwide, mainly in arid regions due to its high resilience. It has been accepted as a model plant because of its characteristics that facilitate scientific research (Li and Brutnell, 2011) and its similar morphology to Panicoideae such as maize (*Zea mays*), sorghum (*Sorghum bicolor*) and sugarcane

(*Saccharum officinarum*) (Brutnell *et al*., 2010). Its tolerance to drought has been extensively studied. It was shown that *S. viridis* (i) recovers from relatively prolonged drought (Luo *et al*., 2009; Luo *et al*., 2011); (ii) makes osmotic adjustments (Luo *et al*., 2011; Saha *et al*., 2016); and (iii) keeps a strong correlation between water use efficiency and plant size (Feldman *et al*., 2018). In (i) and (ii), the authors used complete irrigation withdrawal to prevent plants expressing tolerance to water deficit through a lower growth rate, as demonstrated in (iii). In addition, previous experiments performed by our research group demonstrated that *S. viridis* could not only recover from drought, as demonstrated by Luo *et al*. (2011), but it is capable of retaining growth at a lower rate in drought conditions (paper under preparation). Therefore, a prolonged water deficit with a controlled limit would allow plants to express their tolerance.

In order to better characterize the putative mechanisms involved in the plant tolerance to abiotic stress, we subjected *S. viridis* (A10.1 accession) to long-term osmotic stress and evaluated several morphological and physiological traits. The choice for the A10.1 accession was based on a comparison between physiological plasticity of six accessions of *S. viridis* in response to drought and heat stress that characterized accession A10.1 as extremely tolerant (Saha *et al*., 2016). Since polyethylene glycol (PEG) successively establishes and maintains the tight osmotic potential of solutions, it serves well the purpose of manipulating water availability for plants in any given experimental setup (Nio *et al*., 2019). Consequently physiological parameters of *S. viridis* plants subjected to water deficit with PEG-8000 were evaluated, in response to water availability. Additionally, in order to evaluate the osmotic stress response at the molecular level, five putative *S. viridis* homologs to well-known marker genes involved in the response to drought or water deficit were evaluated. The expression profile of the marker genes was associated with the physiological and morphological changes observed. The new data described in this study can be used as an avenue to understand better plant tolerance mechanisms and to contribute to research on crop improvement.

2. Materials and methods

2.1. Plant growth conditions

This study was conducted using plants of *S. viridis* accession A10.1 kept in a growth room at the Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil

(22°54'10''S; 43°12'27''W; 5.0 m). Plants were grown under a photoperiod of 16/8 h (light/dark), temperature averages of 30±2 °C (light) and 25±2 °C (dark) and artificial illumination using a combination of red, blue, white and orange LED lamps resulting in 750 μmol m^{-2} s^{-1} intensity.

2.2. Experimental set-up

Seeds were germinated in sand inside PET bottlenecks fit into the lid of 2 L pots filled with deionized water to keep the sand always-moist (see Supplementary Fig. S1). A piece of tulle placed at the bottom of the bottlenecks secured the sand while allowing for root growth. After 7 days, each bottleneck containing one plant was transferred to the hydroponics system (Supplementary Fig. S2), consisting of 0.2 L cups containing nutrient solution (one-fourth the concentration of Hoagland; Caisson labs, nº 2) (Rodrigues *et al*., 2020). The nutrient solution was replaced every seven days. The last nutrition solution replacement was with half the normal strength (Hoagland; Caisson labs, nº 2). When the fourth leaf was completely expanded, 15 days post imbibition, treatment was applied using 7.5% of PEG 8000 (about -0.09 MPa). Plants were randomized on the shelves every two days to avoid disparities related to their position. The experiment was carried out twice with the same experimental conditions.

2.3. Chlorophyll a fluorescence analysis

Chlorophyll *a* fluorescence transient was measured using a portable Plant Efficiency Analyzer, Handy PEA (Hansatech, Norfolk, UK). Measurements were taken on intact fully expanded leaves from ten plants from each experimental condition (plants under osmotic stress imposition or control condition), at around 8 A.M. three times during the experiment; on day 3, day 6, and day 10. Measurements were performed 20 min after the adaptation of the leaves to the dark. Fluorescence emission was induced in a 4 mm diameter leaf area by exposing the sample to a pulse of saturating light (3 mmol m^{-2} s^{-1}) and measured during the first second of illumination (10 μs to 1 s). From the transient fluorescence emission curve obtained after the saturating pulse, the intensities determined at 50 μs (initial fluorescence - F_0), 100, 300 μs, 2 (F_J) and 30 (F_I) ms and F_M (maximum fluorescence) were used for the calculation of the parameters established by the JIP Test (Yusuf *et al*., 2010). In addition, the JIP-

test was applied for the analysis and comparison of the OJIP transients using normalizations and subtractions, and difference kinetics from relative variable fluorescence data, calculated according to Yusuf *et al.* (2010). The transients were normalized (general symbol W) as relative variable fluorescence to compare the samples for the events reflected in the OJ, OI and IP phases as $W_{OJ} = (F_t - F_0) / (F_J - F_0)$, $W_{OI} = (F_t - F_0) / (F_I - F_0)$ and $W_{IP} = (F_t - F_I) / (F_P - F_I)$. The difference kinetics ΔW_{OJ} reveals the k-band (at about 300 μs) that is usually hidden (between the steps "O" (50 μs) and "J" (2ms)) and was calculated as $\Delta W = W - W_{ref}$ ("ref" is for untreated). The k-band positive demonstrates an inactivation of the oxygen evolving complex (especially of the Mn-complex) and/or an increase of the functional photosystem II (PS II) antenna size (Yusuf *et al.*, 2010). The transient normalized between the steps "O" (50 μs) and "K" (300 μs), transforms the OK phase to the kinetics $W_{OK} = (F_t - F_0) / (F_K - F_0)$, allowing sample comparisons to be made for energetic connectivity of PS II. The difference kinetics ΔW_{OK} reveals the L-band (at about 150 μs) which when positive demonstrates a better utilization of the excitation energy and higher stability of the system (Yusuf *et al.*, 2010).

2.4. Biometric traits analysis and stomatal conductance

The following morphological parameters were measured from ten plants in each treatment every day: total leaf number; main stem leaf number; tiller number; and exposure angle of the last leaf (measured with a protractor). Shoot and root fresh weights; leaf and root relative water content; shoot and root electrolytic leakage; leaf area; leaf and root osmotic potential; and leaf and root proline content were measured at days 6 and 10. Leaves of five plants per treatment were placed on white paper and photographed for measurement of total area, using ImageJ (NIH) software. These sampling days were chosen based on our previous experiment in which *S. viridis* plants showed severe effects of stress on day 6 and sensitive recovery at day 10 (data not are shown).

Stomatal conductance (g_s) was measured on both leaf surfaces (adaxial and abaxial) using a leaf porometer (SC-1, Decagon Devices, Washington, USA). g_s measurements were taken every day, close to 9 A.M.

2.5. Relative water content, electrolytic leakage, osmotic potential and free proline content

Relative water content (RWC) measurements followed the methodology of Cia *et al*. (2012). Intact fully expanded leaves were collected from five plants in each treatment. Leaves were weighed (FW) and immersed in distilled water for 24 h (at 3°C) for subsequent determination of turgid weight (TW). Leaves were dried in a forced-air oven at 65 °C until a constant weight was reached (approx. 72 h). The RWC was calculated using the formula $RWC = (FW - DW) / (TW - DW) \times 100$; where DW is the dry weight.

Electrolytic Leakage analyzer followed by Liu *et al*. (2015). Fresh leaf and root tissue (0.2 g) were separately placed in Falcon-type tubes containing 20 mL of deionized water. The samples were maintained in a shaker overnight and then the electrical conductivity of the solution (C1) was measured. Soon after, samples were autoclaved at 120 °C for 30 min and when the tubes reached ambient temperature, the conductivity of the solution with the tissue was measured again (C2). Five biological replicates were measured. Electrolytic leakage was calculated using the formula: $EL(\%) = (C1/C2) \times 100$. The electrical conductivity of the solution (C1 and C2) were measured using a conductivity meter (3540 model, Jenway, Staffordshire, UK).

Leaf and root osmotic potential was measured using a dew point potential meter (WP4C, Decagon Devices). Samples were collected and immediately frozen by immersion in liquid nitrogen and then placed in -80°C freeze to posterior analysis. Measurements were done in leaves and roots placed into the WP4C chamber and immediately sealed (Londers *et al*., 2005). Samples were arranged in a way that the chamber's bottom was fully covered. Three repetitions were performed and the arithmetic mean of the readings was considered as the water osmotic value. The instrument was calibrated according to the recommendations by Decagon Devices with KCl solution (KCl 0.5 M; Ψ=-2.19 MPa). Calibration was repeated at the beginning of each reading day.

Free proline content was determined based on the method of Zhang and Huang (2013). Plant samples (0.2-0.25 g) were ground into a powder with liquid nitrogen and homogenized with 3% sulphosalicylic acid, acid ninhydrin, and glacial acetic acid. The resulting mixture was boiled for 45 min in a water bath and then cooled in ice for 30

min. After, toluene was added to the mixture and centrifuged. The optical density of the supernatant solution was measured at 520 nm using a spectrophotometer *Libra* S22 - Biochrom, (England). Three biological replicates were measured. A calibration curve was used to determine the free proline content as µM proline per gram of FW.

2.6. Data analysis

Physiological data were analyzed by Sisvar software (Federal University of Lavras, Lavras, Brazil). The average results from two experimental repetitions were submitted to analysis of variance ($p \leq 0.05$) followed by paired t-test to determine significant differences at each sampling date between untreated and treated plants using PEG 8000. Principal Component Analysis (PCA) was carried out using biometric traits, stomatal conductance, and photosynthesis parameters to investigate variable groupings by PAST 3 version software (PAleontological STatistics).

2.7. Gene expression analysis

For gene expression analysis, roots from control and treated *S. viridis* plants were harvested at day 3, day 6, and day 10. Samples were stored at -80°C and later ground in liquid nitrogen with mortar and pestle. Roots of three plants were ground together to form one biological replicate and the procedure was repeated three times to obtain three biological replicates. Ground tissue (0.15 g) was used for RNA extraction using the SV Total RNA Isolation System from Promega®. Concentration and purity of the RNA samples were measured using Nanodrop 2000 (Thermo Scientific). Synthesis of the complementary DNA strand (cDNA) was performed using 2 µg of total RNA by adding 50 µM of Oligo dT primer, 10 mM of dNTPs, First-Strand 5X buffer, 20 mM of DTT and 200 units of SuperScriptTM III enzyme (Invitrogen), according to the manufacturer's instructions. The cDNA was stored at -20°C to be further used in qPCR analyses.

The genes chosen for expression analysis were selected based on previous studies performed by our group. The first study identified drought marker genes in *S. viridis* exposed to two different water deficit conditions (Rodrigues *et al*., 2020); whereas the second study used transcriptome analysis of roots of *S. viridis* exposed to cycles of dehydration in which was possible to identify late-response genes (Rodrigues

et al., unpublished data). Based on these two studies, we selected five genes to be evaluated by qPCR. Primers were designed with an annealing temperature of 60°C using the Primer3Plus tool (Untergasser *et al.*, 2007). The primer sequences and gene codes are listed in Supplementary Table S1. Quantitative real-time PCR (qPCR) was performed using the 7500 Fast Real-Time PCR detection system (Applied Biosystems), PlatinumTM Taq DNA polymerase (Invitrogen) and SYBR Green as a dye to monitor the amplification of DNA. ROX reference dye (Invitrogen) was used for the correction of the sample data based on background fluorescence. Raw amplification data were analyzed using the Real-time PCR Miner (Zhao and Fernald, 2005) to identify the exponential phase of the reaction and calculate the Ct (threshold cycle). Relative gene expression was calculated using Relative Expression Software Tool - 384 (REST - 384) (Pfaffl *at al.*, 2002), comparing control and treated samples and testing for significance with a Pair Wise Fixed Reallocation Randomization Test. Two constitutive genes (*Seita.1G130900* e *Seita.3G037700*), previously identified as good reference genes during water deficit stress, were used for calculation of normalization factor. Relative gene expression results are presented as log2Fold-Change (log2FC), in which the fold change is the ratio between expression levels from treated and control samples. Results were plotted in a heatmap, constructed in R using the latticeExtra package (Sarkar and Andrews, 2016).

3. Results

3.1. Principal component analysis (PCA)

Principal component analysis (PCA) showed a comprehensive understanding of the influence water limitation had on the analyzed parameters and identified the same groupings at 6 and 10 days after stress imposition (Figs. 1 and 2). The first and second components (PC1 and PC2) explained, respectively, 72.5 and 10.7% at day 6 and 71.6 and 14.5% at day 10 of the total variation among individuals.

At day 6 under stress (Fig. 1), all physiological parameters evaluated, except RWC, were responsible for grouping the individuals exposed to 7.5% of PEG 8000. Control plants were also grouped by higher root electrolytic leakage values in comparison to treated plants. At day 10, abaxial stomatal conductance and maximum

photochemical efficiency were not responsible for grouping, while root RWC values were lower in the plants treated with PEG 8000 (Fig. 2).

3.2. Biometric traits, stomatal conductance, electrolytic leakage and proline

The prolonged use of 7.5% of PEG 8000 had a direct impact on the biometric traits, such as leaf and tiller number, while reducing plant growth rate (Fig. 3 and Table 1). Treated plants had no tillers, exhibited yellowing mainly of the first leaves with necrotic areas, and reddening from the base to the apex of the stem (Fig. 3). Despite the slower emergence of new leaves with a smaller area, they were of color and turgidity close to that of control plants. Nevertheless, plants exposed to PEG had a severe reduction in biomass (Table 2) which affected the shoot to root ratio after ten days (2.67 for control versus 1.84 for treated plants). PEG treatment led to a reduction of ~85% and ~94% in shoot fresh weight (SFW) after 6 and 10 days, respectively (Fig. 3 and Table 2). The exposure angle of the last leaf of stressed plants was higher than the control plants between the days 6-9.

A significant difference between control and stressed plants was observed for stomatal conductance between both leaf surfaces (adaxial and abaxial). The adaxial stomatal conductance was, on average, twice as high as the abaxial side for both treatments. The osmotic stress caused a progressive decrease in abaxial stomatal conductance from day 3 to day 10 while on the adaxial side it started the decrease one day later and continued to declin to day 10 (Table 1). The difference between control and treated plants was smaller on day 10 (about 27%). Root electrolytic leakage of the plants subjected to the PEG treatment was lower than on control plants on both days (6th and 10th), while shoot electrolytic leakage was equivalent. The difference for root proline content between treated and untreated plants was not significant ($p>0.05$). Leaf proline content showed a significant increase in the treated plant on both days.

3.3. Chlorophyll a fluorescence transients

Chlorophyll *a* fluorescence transients of the dark-adapted leaves of *S. viridis* plants are shown on a logarithmic time scale from 50 μs up to 1 s on day 3 (Fig. 4A), day 6 (Fig. 5A) and day 10 (Fig. 6A) after imposition of stress. The typical OJIP shape (the $O_{50\mu s}$, J_{2ms}, I_{30ms}, and P_{1s} steps are marked in the plot) was affected by the osmotic

stress mainly between O-I steps with similar maximum variable fluorescence (F_M−F_0=F_V), demonstrating that the control and treated plants were photosynthetically active.

Some functional and structural parameters of the photosynthetic behavior of *S. viridis* subjected to osmotic stress, on three collecting times, were deduced by the JIP-test and shown in Fig. 4D-F (energy distribution in the photosynthetic apparatus). Treated plants parameters were normalized with untreated plants. The sequential energy transduction indicated by Reaction Centres (RC) showed an increase in the energy fluxes for (light) absorption [ABS/RC; which measures the apparent antenna size] and in the energy dissipated as heat per RC (DI_0/RC) on the third day of stress and intensified until the sixth day. In the opposite way, ABS/RC and DI_0/RC decreased during the remaining days up to day 10. Trapping flux (leading to quinone A (Q_A) reduction) per RC (TR_0/RC) and electron trapping (ET_0/RC; which measures the energy trapping flux per active RC, able to lead a Q_A reduction) also increased at day 6. Alternatively, the electron flux reducing end electron acceptors at the PSI acceptor side (RE_0/RC) decreased at day 3 and day 10. On the three sampling days, a decrease in quantum yield for electron transport (φ_{Eo}) was observed, reflecting a decrease in efficiency with which an electron from the intersystem electron carriers moves to reduce end electron acceptors at the PSI acceptor side (δ_{Ro}). The efficiency for electron transport (ψ_{Eo}) decreased on day 3 and day 10 while the maximum quantum yield of PSII (F_V/F_M) decreased until day 6 and did not show a difference on day 10. All three days showed a decrease in both the performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors (PI_{abs}) and the performance index (potential) for energy conservation from exciton to the reduction of PSI end acceptors (PI_{total}).

Fluorescence data were normalized between the steps O (50 μs) and I (30 ms) and presented as relative variable fluorescence W_{OI} = (F_t − F_0) / (F_I −F_0) vs. time (logarithmic time scale from 50 μs up to 30 ms). We observed different curve behavior between control and treated plants on the three sampling days (3, 6, and 10 days after stress imposition) (Fig. 5A-C) with considerable recovery on the 10th day. Data normalization allows distinguishing the events from exciton trapping by PSII up to plastoquinone (PQ) reduction. A slight decrease in the sequence of events from the PSI-

driven electron transfer to the end electron acceptors on PSI acceptor side, starting at PQH_2 (plastoquinol) (W_{IP}) (Fig. 5D-F; with its respective WOI graphs insert) was perceived in the third day and became accentuated on days 6 and 10.

The fluorescence data normalized between the steps O (50 μs) and K (300 μs), and plotted with the difference kinetics $\Delta W_{OK} = W_{OK(osmotic\ stress)} - W_{OK(control)}$ in the 50–300 μs time range revealed the L-band (at about 150 μs), an indicator of the energetic connectivity of the PS II units. The presence of a positive L-band was observed on the 3rd and 6th days (Fig. 6A-B). However, on day 10 no difference was observed in the connectivity results (L-band) (Fig. 6C). The fluorescence data normalized between the steps O (50ms) and J (2 ms) and plotted with the difference kinetics $\Delta W_{OJ} = W_{OJ(osmotic\ stress)} - W_{OJ(control)}$ in the 50 μs–2 ms time range revealed the presence of positive K-band mainly on day 6 (Fig. 6D-F).

3.4. Gene expression analysis

To understand the molecular mechanisms associated with *S. viridis* response to long-term water deficit conditions, qPCR analysis was used to evaluate the expression of the selected genes *SvLEA*, *SvDREB1C*, *SvPIP2-1*, *SvHSP20* and *SvP5CS2* (Supplementary Table S1) on three key sampling days (days 3, 6 and 10) during the stress treatment. The selected genes are putative *S. viridis* homologs to genes previously described as involved in several biochemical, physiological and cellular processes induced by water deficit, suggesting that they are functional orthologs of Arabidopsis genes. Therefore, they can be considered as marker genes for abiotic water condition in *S. viridis*. *SvHSP20* encodes a heat-shock protein that acts as a molecular chaperone, ensuring the correct folding of newly synthesized polypeptide chains and proteins damaged by stress (Yan *et al.*, 2017). The gene *SvP5CS2* encodes an enzyme necessary for the biosynthesis of proline and it is associated with osmoregulation (Mattioli *et al.*, 2009). The *SvDREB1C* gene encodes a transcription factor responsive to water deficit and *SvLEA* encodes a dehydrin protein, both highly involved in the response to abiotic stress (Ito *et al.*, 2006; Liu *et al.*, 2017). The gene *SvPIP2-1* is a water channel gene, regulating the transport of water across cell membrane encoding an aquaporin protein, which acts as a water channel during water stress (Afzal *et al.*, 2016). On day 3, all the genes were significantly up-regulated, except for *SvP5CS2* which was down-regulated

in comparison to the control condition (Fig. 7). Genes *SvLEA*, *SvDREB1C*, *SvPIP2-1*, and *SvP5CS2* continued so at days 6 and 10 (Fig. 7). The expression of *SvHSP20* was not differentially regulated at days 6 and 10 (Fig. 7).

4. Discussion

Crop productivity can be severely affected by drought stress, the most prevailing abiotic factor worldwide (Shi *et al*., 2018). Plant tolerance to drought is fundamentally related to the ability to efficiently access and use soil water (Richards *et al*., 2010). In this study, we used the C_4 model plant species *S. viridis* to investigate its response to a long-term osmotic stress condition. For that, *S. viridis* was subjected to water limitation for 10 days, and plant morphological and physiological responses were characterized.

S. viridis plants reacted to osmotic stress caused by PEG-8000 treatment in the hydroponics system by slowing down their growth. All the main morphological traits evaluated on day 3, and day 10, and during the experiment, such as leaf area and shoot weight, were negatively affected by osmotic stress (Figs. 1 and 2; Tables 1 and 2). In rice (*Oryza sativa*) and barley (*Hordeum vulgare*), decrease of leaf area and weight are the first traits affected by drought, which could be considered as a tolerance mechanism, because it reduces transpiration and demand for water (El-Samad *et al*., 2019; Chaves *et al*., 2009). Thus, plant-sustained growth in adverse environments is intrinsically associated with water use, as reported in the interspecific *S. italica* × *S. viridis* recombinant inbred (Feldman *et al*., 2018). Similarly, slower growth allows plants to divert assimilates and energy into protective molecules in order to face stress and to maintain root growth, thus, improving water acquisition. In addition, with fewer leaves, the treated plants exhibited a greater exposure angle of the last leaves compared to the control that kept them upright to avoid self-shading.

In the same way, shoot and root development can be associated with drought tolerance as an adaptive trait (El-Samad *et al*., 2019). *S. viridis* decreased the shoot to root ratio suggesting higher investment on root protection mechanisms. In soil conditions, a deeper root system improves plant acclimation during drought offering greater capacity for water uptake. This in turn would keep high water status in leaves allowing a better performance during times of drought. In this study, in the PEG treated plants, the higher investment in roots associates with lower shoot area allowed for the

maintenance of leaf RWC close to control plants (Table 1). Such a strategy may help explain high tolerance to drought displayed by *S. viridis*.

Increased electrolytic leakage is a well-known stress indicator used to assess the severity of drought-induced oxidative stress (Rezayian *et al*., 2020). Nevertheless, here we consistently measured a decrease in electrolyte leakage from the roots of stressed plants. Although counter-intuitive, a reduction in electrolytic leakage was reported in leaves of soybean and hulless barley under drought stress (Rezayian *et al*., 2020; Gan *et al*., 2015) and brown mustard under salt stress (Zeng *et al*., 2011) after an application of nitric oxide. Nitric oxide has been associated with signaling in biotic and abiotic responses of plants to stress (Domingos *et al*., 2015), repairing and protecting the cell membrane to alleviate the injury in the cell membrane system. In this study, other factors are potential candidates for membrane protection and the consequent reduction in electrolytic leakage. For instance, proline accumulation or dehydrin production (*SvLEA* gene up-regulation). To the best of our knowledge, crops that lower electrolytic leakage under stress without the action of an external agent have not been reported. Sandhya *et al*., 2010, demonstrated protection of membrane and prevention of ion leakage giving tolerance to plants under drought stress, in maize inoculated with *Pseudomonas* spp.

Although the osmotic stress condition with 7.5 % PEG 8000 could be considered mild (about -0.09 MPa), it caused, in the relative long-term, a severe reduction in stomatal conductance and, consequently, decrease in the photosynthesis parameters (Table 1 and Figs 4-6). The production of photo-assimilates, such as sugar, plays an important role in stress response, modulating growth (Duque *et al*., 2013). In moderate PEG (about -0.55 MPa), *S. viridis* photosynthesis exhibited damage in the first hours and further negative effects until 7 h (Rodrigues et al., 2020). In another study, rapidly and slowly imposed water deficit caused different effects on enzymes involved in photosynthesis of *Setaria sphacelata* (Marques da Silva and Arrabaca¸ 2004). Here, the use of PEG was an appropriate method to study the effects of water deficit, affecting physiological indicators of stress, such as photosynthesis, and allowing a slower water loss, closer to field conditions (Rodrigues *et al*., 2020; Nio *et al*., 2019).

The reduction in F_V/F_M value (about 25 %) observed on day 6 suggests inhibition of the trapping probability of PS II under water limitation. The increase

observed in ABS/RC (about 100 %) and DI_0/RC (about 150%) under drought reflect photo-inhibition (Luo *et al.*, 2009). As the increased rate was higher in DI_0/RC than in the ABS/RC, a fraction of RCs may have been inactivated and transformed into non-Q_A-reducing centers. In the same way, ABS/RC increase followed by an increase of TP_0/RC (about 50 %) suggests that the functional antenna (that supplies excitation energy to active RCs) has increased in size. In this case, energy supply to the active RCs from the antenna associated with inactive RCs could be related to the increase in the size of a functional antenna. However, TP_0/RC did not increase ABS/RC to the same rate (50 to 100 % of the increase) showing that some absorbing antenna chlorophylls did not feed the active RCs and dissipated their excitation energy by heat (Yusuf *et al.*, 2010). Similarly, the presence of positive K-band on day 6 (Fig. 6E) is in agreement with both an inactivation of the oxygen-evolving complex (OEC, Fig. 4E) and an increase of the functional PS II antenna size (Fig. 4E). Therefore, from our results, it is possible to suggest that the transformation of RCs to inactive centers could due to inactivation of the oxygen-evolving complex and to their structural transformation to heat sinks in *S. viridis* on day 6 (Yusuf *et al.*, 2010). In addition, the presence of a positive L-band (Fig. 6A-B) indicates a loss of energetic connectivity, inefficient consumption of the excitation energy, and instability of the system on the 3rd and 6th days.

On day 10, plants maintained under osmotic stress did not exhibit inhibition of the trapping probability of PS II, showing recovery on F_V/F_M in comparison to plants on day 6 (Fig. 4). Similarly, despite RCs inactivated (DI_0/RC and ABS/RC increases) functional antenna did not follow an increase in size, as it was observed on day 6 (no difference in TP_0/RC) (Fig. 4). Besides this, no difference was observed in energetic connectivity (no difference in L-band) (Fig. 6C), which shows a better utilization of the excitation energy and higher stability of the system when compared to the 6th day. It has been demonstrated that *S. viridis* presents the capability to recover from drought, mainly in their photosynthesis and stomatal conductance; which may contribute to the use of water as efficiently as possible (Luo *et al.*, 2009). In this study, besides no difference in F_V/F_M on day 10, parameters as gs, PI_{abs} and PI_{total} were kept below the control plants despite demonstrating a slight increase from day 6 to day 10. PI_{total} indicates a decrease in the functionality of the electron transport chain of plants caused by osmotic stress and

is the most sensitive parameter of the JIP-test. It incorporates parameters evaluated from the fluorescence transient OJIP such φ_{Po} (using F_0 and F_M), δ_{Ro} (using variables fluorescence) and RC/ABS ratio (using φ_{Po} and variables fluorescence) while F_V/F_M is only sensitive to the ratio F_0/F_M (Yusuf *et al.*, 2010). Thus, PI_{total} expresses any change in the OJIP transient test and it is closely related to plants performance, such their growth or survival under stress conditions. Plants response and tolerance mechanisms involved in abiotic stress are complex but based on the data evaluated in this study a correlation was found between PI_{total} and physiological traits, such as the plant biometrics (Table 1).

The evaluation of the molecular mechanisms associated with *S. viridis* response to long-term osmotic stress conditions could help to understand the changes in physiological traits. To investigate the expression level of *S. viridis* marker genes, previously associated with drought or abiotic stress, we performed qPCR analyses in roots from *S. viridis* under osmotic stress on days 3, 6, and 10. *SvLEA* gene, a putative *S. viridis* homolog to dehydrin protein, was significantly up-regulated at all time-points, with a higher expression level on day 3 (Fig. 7). Dehydrin protein has multiple roles, such as membrane protection, cryoprotection of enzymes, and protection from reactive oxygen species (Liu *et al.*, 2017). Dehydrin gene expression was associated with a lower level of electrolyte leakage in plants under cold stress (Liu *et al.*, 2017) and in a *Panicum virgatum* cultivar with higher drought tolerance (Liu *et al.*, 2019). The up-regulation of *SvLEA* observed by the qPCR analyses is in agreement with the lower root electrolyte leakage of the *S. viridis* plants under osmotic stress caused by the PEG (Table 2). Additionally, we evaluated the expression of *SvHSP20*, a putative *S. viridis,* heat shock protein homolog, involved in the protection of proteins against denaturation under several types of abiotic stress, thus improving stress tolerance (Yan *et al.*, 2017). qPCR analysis indicated that *SvHSP20* was up-regulated only at the early stress (day 3), but had no significant expression on days 6 and 10 (Fig. 7). The expression profile of *SvHSP20* could substantiate our observations of improvements in some photosynthesis parameters (like F_V/F_M and PI_{abs}) and stomatal conductance by mitigating the effects of dehydration (Table 1 and Fig. 4). Similar responses were observed in *P. virtagum* and rice plants under abiotic stress conditions (Yan *et al.*, 2017; Liu-Ming *et al.*, 2020). In transgenic rice, plants overexpressing *OsHSP20* under heat and salt stress increased root

size and germination rates (Liu-Ming *et al*., 2020). In addition, the accumulation of *PvHSP20* in *Panicum virgatum* L. facilitated its adaptation under hot and dry conditions (Yan *et al*., 2017).

Proline accumulation is an important response of plants under osmotic stress, affecting several plant cellular and molecular aspects (Amini *et al*., 2015). Proline is associated with membrane protection (Ashraf & Foolad, 2007). Proline accumulation in plants subjected to drought conditions could either mean (i) an adaptive property to drought tolerance, acting as protection (Júnior *et al*., 2020; Queiroz *et al*., 2011), or (ii) a symptom of stress (Cia *et al*., 2012). In the first case, the accumulation of proline could be contributing, among others, to the stabilization of biological membranes, acclimation of photosynthetic apparatus, scavenging free radicals, recovery of stomata from the water shortage and osmoregulation (Luo *et al*., 2011). In addition, proline accumulation could act as a source of energy following stress (Júnior *et al*., 2020). In this study, we evaluated the expression of *SvP5CS2*, a putative *S. viridis* homolog gene associated with the proline biosynthesis pathway (Mattioli *et al*., 2009). The expression of *SvP5CS2* in roots of *S. viridis* was down-regulated on day 3, despite a small, but significant, up-regulation on days 6 and 10 (Fig.7). The up-regulation of *SvP5CS2* gene in root and leaves of *S. viridis* under a most severe water deficit (- 0.55 MPa) was observed for a short period with PEG treatment (1 to 7 h) (Rodrigues *et al*., 2020). Szabados and Savouré (2010) suggested that the *AtP5CS2* gene may function as housekeeping gene and *AtP5CS1* gene would be responsible for higher proline biosynthesis under water deficit stress. In spite of our previous results that indicated a transcriptional regulation of *AtP5CS2*, it would be interesting to study *AtP5CS2* gene regulation with other water deficit methods. Consistent to the low *SvP5CS2* up-regulation on days 6 and 10 the evaluation of proline content in roots presented no significant difference on days 6 and 10, when compared to the roots of *S. viridis* under control conditions (Table 2).

The increase in leaf proline accumulation on days 6 and 10 (Table2) may be due to lower demand for amino acids for protein synthesis (Silveira *et al*., 2003). Another putative explanation could be enhancing proline synthesis, as indicated in the work with *S. viridis* under higher osmotic stress (Rodrigues *et al*., 2020). Nevertheless, leaf proline levels were well correlated with stomatal conductance, which can indicate a role of

proline in the recovery of stomata and the photosynthetic apparatus by the storage of water (Luo *et al*., 2011). In this study, despite lower stomatal conductance in the treated plants, compared to control plants on day 10, there was an increase of 38% in relation to day 6 with the treated plants, while control plants showed a 40% decrease (Table 2). This decrease in stomatal conductance of control plants is possibly related to proximity to the moment of inflorescence emergence (25 days after imbibition, Junqueira *et al*., 2020). Proline accumulation is described as having an additive effect to abscisic acid (ABA) in stomatal closure, through the interaction of these substances with cytokinin (Klein and Itai, 1989). Thus, proline accumulation correlates with stomatal resistance (Klein and Itai, 1989). In this case, the proline accumulation observed could have favored a recovery of photosynthetic apparatus of *S. viridis* plants under osmotic stress, avoiding water loss (Dichio *et al*., 2006). In addition, Saha *et al*. (2016) and Luo *et al*. (2011) reported proline accumulatio in *S. viridis* under drought. However, this accumulation was observed in low RWC plants. The authors explain their results suggesting an osmotic adjustment by reduced osmotic potential, even though they did not measure it. Our data show accumulation in leaf proline with osmotic stress, which is in accordance with these authors. However, here in this study it is associated with the fact that neither leaf RWC nor osmotic potential were reduced in relation to the control (Table 2), therefore discarding the possibility of osmotic adjustment.

The differences observed between our data and those of other authors in leaf RWC, at least partly, could be due to the specific kind of stress that was imposed. Different from the previous studies where the imposed a decrease in the water potential of soil was by complete irrigation withdrawal, we induced a controlled reduction of the osmotic potential of the nutrient solution, using PEG 8000 (about -0.09 MPa). Experimentally applying stress imposition at different rates is important to try and study the counter reaction of plants (Duque *et* al., 2013; Marques da Silva and Arrabaca¸ 2004). Thus, the use of PEG methods may have allowed plants to express tolerance to water deficit through lower growth, e.g., reducing losses by transpiration. Feldman *et al*., 2018 demonstrated that plant size and water use are strongly correlated. Our data are in accordance with these authors; where they found a decrease in plant size under partial irrigation withdrawal in soil.

Plant response and tolerance to water deficit involve hormonal signaling promoted by abscisic acid (ABA) (Lata and Passad, 2011). ABA signaling could be related to the regulation of stomatal aperture and reduced plant growth; since reducing water loss through transpiration, decreases the photosynthetic rate and improves osmotic stress tolerance (Ito *et al*., 2006). We evaluated the expression of the gene *SvDREB1C* of *S. viridis*, a putative homolog of *AtDREB1C*, a marker gene responsive to ABA and water-deficit condition in *A. thaliana* (Ito *et al*., 2006). *SvDREB1C* was up-regulated during the whole experiment (days 3, 6, and 10) (Fig. 7) and that expression profile may be associated with ABA-dependent signaling in *S. viridis* under water deficit promoted by the osmotic stress treatment. Interestingly, previous studies have shown that the gene *SvDREB1C* was also up-regulated in root from *S. viridis* plants submitted to a most severe osmotic stress, using the higher concentration from PEG (-0.55 MPa) (Rodrigues *et al*., 2020). Water deficit could trigger increased levels of ABA in *S. viridis* plants (Saha *et al*., 2016). In transgenic rice plants, for example, besides getting more tolerant to drought, the overexpression of the *OsDREB1* gene was related to growth retardation even in normal conditions (Ito *et al*., 2006). In the same way, transgenic *Salvia miltiorrhiza* increased their survival and showed a reduction in growth when overexpressing *AtDREB1C* gene under severe drought (Wei *et al*., 2017).

Aquaporin has been related to improvement drought tolerance and water movement within plants, allowing an increase in stomatal conductance under abiotic stress in several species, including *S. viridis* (Saha *et al*., 2016). We measured the expression of an aquaporin gene (*SvPIP2-1*) that facilitates the transport of water across cellular membranes (Afzal *et al*., 2016; Alexandersson *et al*., 2010). qPCR analysis showed that *SvPIP2-1* was highly expressed on day 3 and kept its up-regulation on days 6 and 10 (Fig. 7), corroborating with the data obtained by the physiological parameters, such maintenance in RWC on days 6 and 10 (Table 2.) and improvement of stomatal conductance on day 10 (Table 1) in *S. viridis* plants subjected to osmotic stress.

5. Conclusion

Based on the physiological and morphological parameters associated with the expression profile of key marker genes evaluated in this study, it is possible to suggest that *S. viridis* showed tolerance to long-term osmotic stress. The evaluation of the

expression of well-known marker genes responsive to abiotic stress contributed to the understanding of the mechanisms involved in *S. viridis* response to osmotic stress. Together, our results highlighted an improvement in physiological traits of *S. viridis* (accession A.10) under long-term osmotic stress and the new data described here will be a useful for further studies in Panicoideae C_4 plants.

Author contribution

D.C.V., S.M.M. and J.T.L. carried out the plant experiments and performed the physiological and molecular investigations. S.M.M. and J.T.L. performed the gene expression analyses. D.C.V. and S.M.M. wrote the manuscript. A.M. revised the language. All the authors revised and approved the final version of the manuscript.

Acknowledgments

This work is part of the doctoral thesis of D.C.V. in Plant Biotechnology (Programa de Pós-Graduação em Biotecnologia Vegetal e Bioprocessos, Universidade Federal do Rio de Janeiro, Brazil), supported by a scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) (141802 / 2016-2). S.M.M. was supported by a Junior postdoc scholarship (Pós-doutorado Junior - PDJ) from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) (159178 / 2018-5). We thank Professor Daniel Fonseca de Carvalho that made the WP4C machine available for the osmotic potential analysis and Filipe Valeriano for technical support.

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Tables

Table 1. Main stem leaf number, total leaf number, number of tillers, last leaf exposure angle (°), adaxial and abaxial stomatal conductance (mmol m^{-2} s^{-1}) of *Setaria viridis*. A paired t-test determined significant differences at each sampling date between untreated and treated plants using PEG 8000. Significance level indicated by * (P<0.05) or ** (P<0.01). (Means ± standard deviation; n = 10-20).

Traits	Days after stress imposition Control/ 7.5% of PEG 8000									
	1	2	3	4	5	6	7	8	9	10
Main stem leaf number	3.1±0.3/ 3±0	3.5±0.7/ 3.3±0.5	4.1±0.5/ 3.7±0.7	4.7±0.7/ 3.9±0.7**	5.3±0.5/ 4.2±0.8**	5.8±0.7/ 4.6±0.8**	6.3±0.8/ 4.9±1**	7.3±0.5/ 5.8±0.4**	7.4±0.7/ 5.3±1**	7.7±0.5/ 5.8±0.8**
Total leaf number	3.8±0.4/ 3.7±0.5	4.4±0.9/ 3.9±0.3	6.3±1.7/ 4.8±0.9**	8.4±2.5/ 5.1±1.1**	10.6±2.9/ 5.3±1.2**	12.4±3.5/ 5.1±2.2**	15.4±3.7/ 4.7±1.9**	23.7±3.3/ 5.3±0.7**	23.9±6.8/ 4.1±1.2**	27.9±6.5/ 5.3±2.3**
Tillers number	0±0/ 0±0	0.3±0.7/ 0±0	1.1±0.9/ 0.1±0.4**	1.8±0.9/ 0.2±0.5**	2.3±0.7/ 0.3±0.6**	2.9±0.8/ 0.3±0.6**	3.4±0.7/ 0.1±0.5**	5.7±1.5/ 0.3±0.7**	5.6±1.9/ 0.2±0.5**	7.3±2/ 0.1±0.5**
Exposure angle of last leaf	94±35/ 91±24	112±13/ 91±23	100±19/ 93±18	93±17/ 97±24	82±19/ 95±18	68±25/ 90±14*	57±22/ 93±15**	64±15/ 88±15**	37±23/ 91±12**	61±22/ 77±21
Adaxial stomatal conductance	173±69/ 144±58	168±57/ 106±36**	187±74/ 73±18**	206±46/ 57±22**	212±59/ 53±20**	187±48/ 60±22**	162±45/ 66±26**	148±31/ 61±26**	143±27/ 73±32**	113±26/ 83±18**
Abaxial stomatal conductance	67±24/ 54±25	63±23/ 54±29	76±28/ 48±29*	79±14/ 45±16**	82±22/ 34±14**	74±22/ 30±13**	71±18/ 31±7**	56±19/ 27±4**	56±21/ 28±6**	56±23/ 35±9*

Table 2. Shoot and root fresh weight (FW, g), leaf and root relative water content (%), leaf and root Electrolytic leakage (%), leaf area (cm^2), leaf and root osmotic potential (MPa) and leaf and root proline content ($\mu M\ gFW^{-1}$) of *Setaria viridis* to control and stresses plants at 6 and 10 days after stress imposition. A paired t-test determined significant differences at each sampling date between untreated and treated plants using PEG 8000. Significance level indicated by * ($P<0.05$) or ** ($P<0.01$). (Means ± standard deviation; n = 3-5).

Traits	***Days after stress imposition***			
	6		10	
	Control	PEG 8000 (7.5%)	Control	PEG 8000 (7.5%)
Shoot fresh weight	0.90±0.09	0.14±0.04**	2.87±0.60	0.18±0.07**
Root fresh weight	0.43±0.09	0.08±0.04**	1.12±0.32	0.10±0.05**
Leaf RWC	0.96±0.03	0.89±0.19	0.86±0.04	0.87±0.07
Root RWC	0.93±0.04	0.84±0.08	0.92±0.03	0.80±0.06**
Leaf Electrolytic leakage	3.84±0.84	23.63±26.63	2.78±0.43	10.96±9.02
Root Electrolytic leakage	57.62±5.1	32.62±4.1**	41.99±5.5	22.51±8.2**
Leaf area	28.7±5.5	4.8±1.7**	75.9±18	6.2±4**
Leaf osmotic potential	-0.8±0.0	-1.2±0.6	-0.8±0.1	-0.6±0.1
Root osmotic potential	--	--	-0.5±0.1	-0.5±0.1
Leaf proline content	54±20	472±185*	99±47	1166±723*
Root proline content	121.2±63	95.7±66	114.6±13	84.6±14

Figures

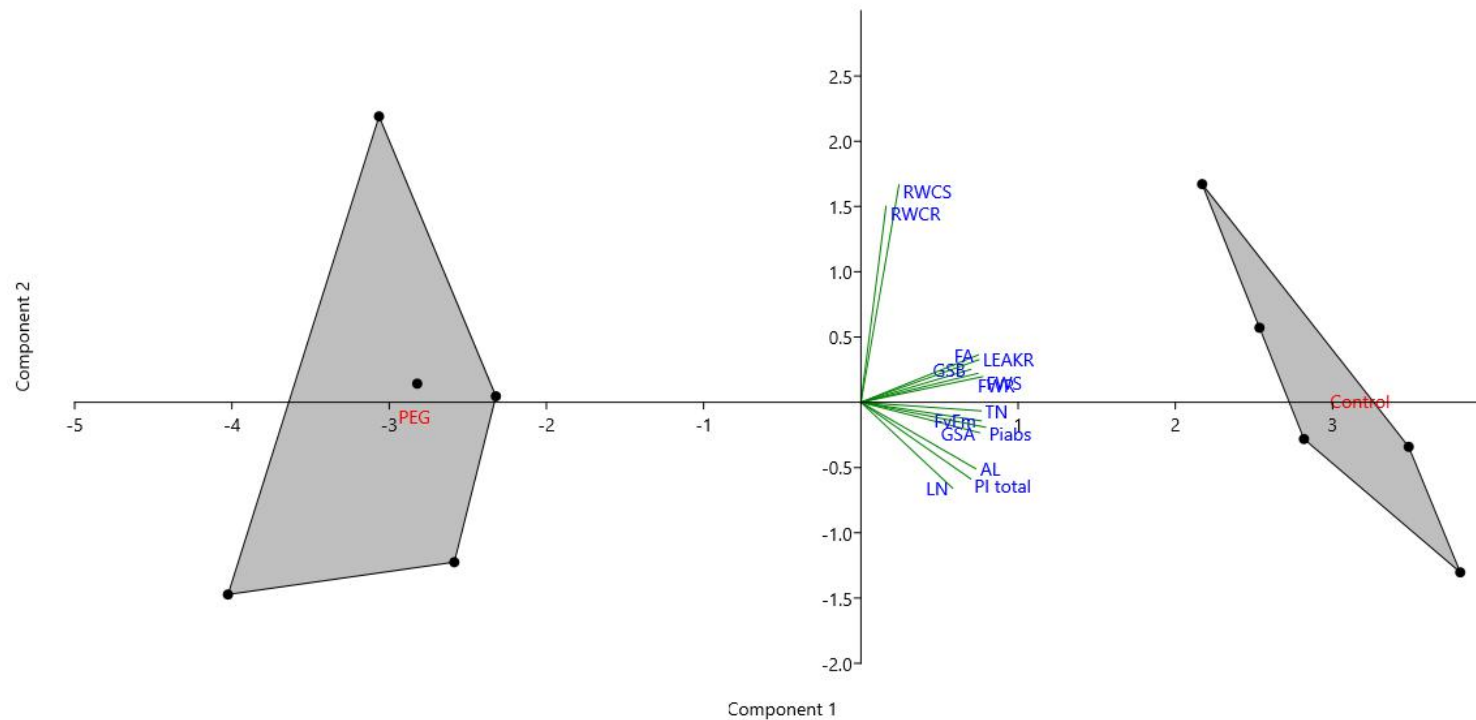


Fig. 1. Principal component analysis (PCA) of *S. viridis* exposed to 7.5% PEG 8000 on day 6 after oxidative stress imposition. Axes are on an eigenvalue scale. RWCS: Relative water content in shoot; RWCR: Relative water content in root; FA: Foliar area; LEAKR: electrolytic leakage in root; GSB: stomatal conductance in abaxial surface; GSA: stomatal conductance in adaxial surface; FWS: shoot fresh weight; FWR: root fresh weight; TN: Tillers number; AL: total number of leaves; LN: Number of leaves of the main stem; ; F_V/F_M: maximum photochemical efficiency; Pi_{abs} and Pi_{total}: performance indexes of chlorophyll *a* fluorescence (JIP-test). (n = 5).

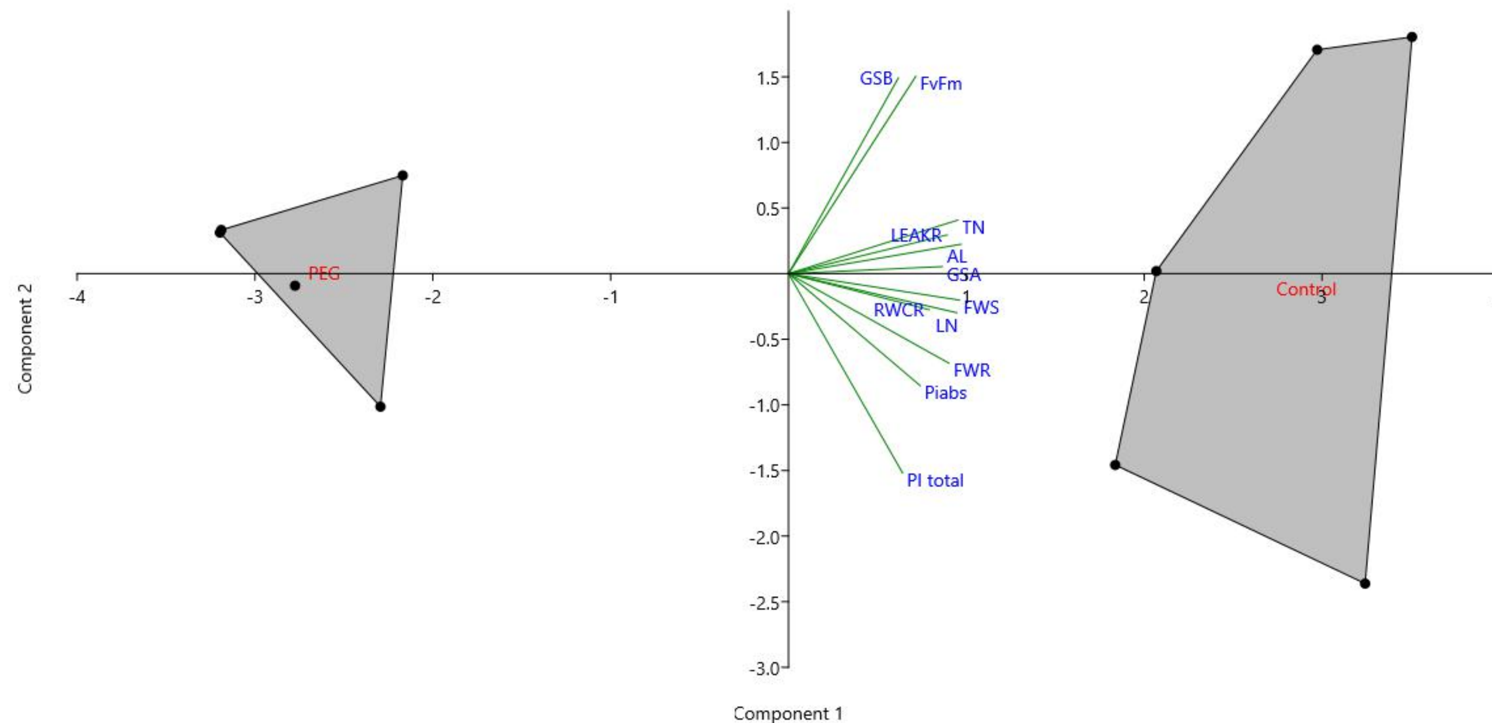


Fig. 2. Principal component analysis (PCA) of *S. viridis* exposed to 7.5% PEG 8000 on day 10 after oxidative stress imposition. Axes are on an eigenvalue scale. RWCR: Relative water content in root; LEAKR: electrolytic leakage in root; GSB: stomatal conductance in abaxial surface; GSA: stomatal conductance in adaxial surface; FWS: shoot fresh weight; FWR: root fresh weight; TN: Tillers number; AL: total number of leaves; LN: Number of leaves of the main stem; F_V/F_M: maximum photochemical efficiency; Pi_{abs} and Pi_{total}: performance indexes of chlorophyll *a* fluorescence (JIP-test). (n = 5)

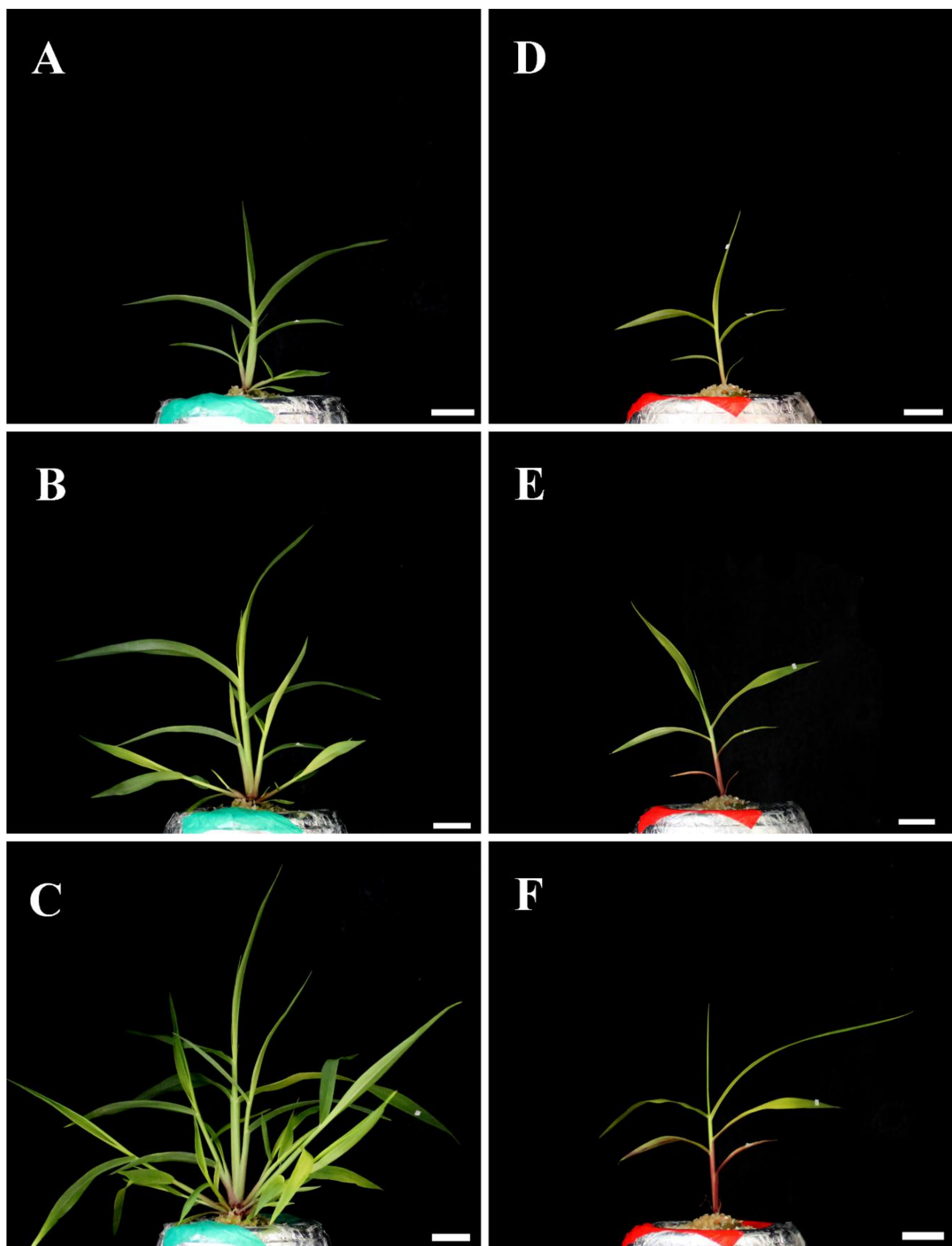


Fig. 3. Plants of *Setaria viridis* throughout experimentation. Untreated (A-C) and exposed to 7.5% PEG 8000 plants (D-F) on day 3 (A and D), 6 (B and E) and 10 (C and F) respectively under stress. Scale Bars: 2 cm.

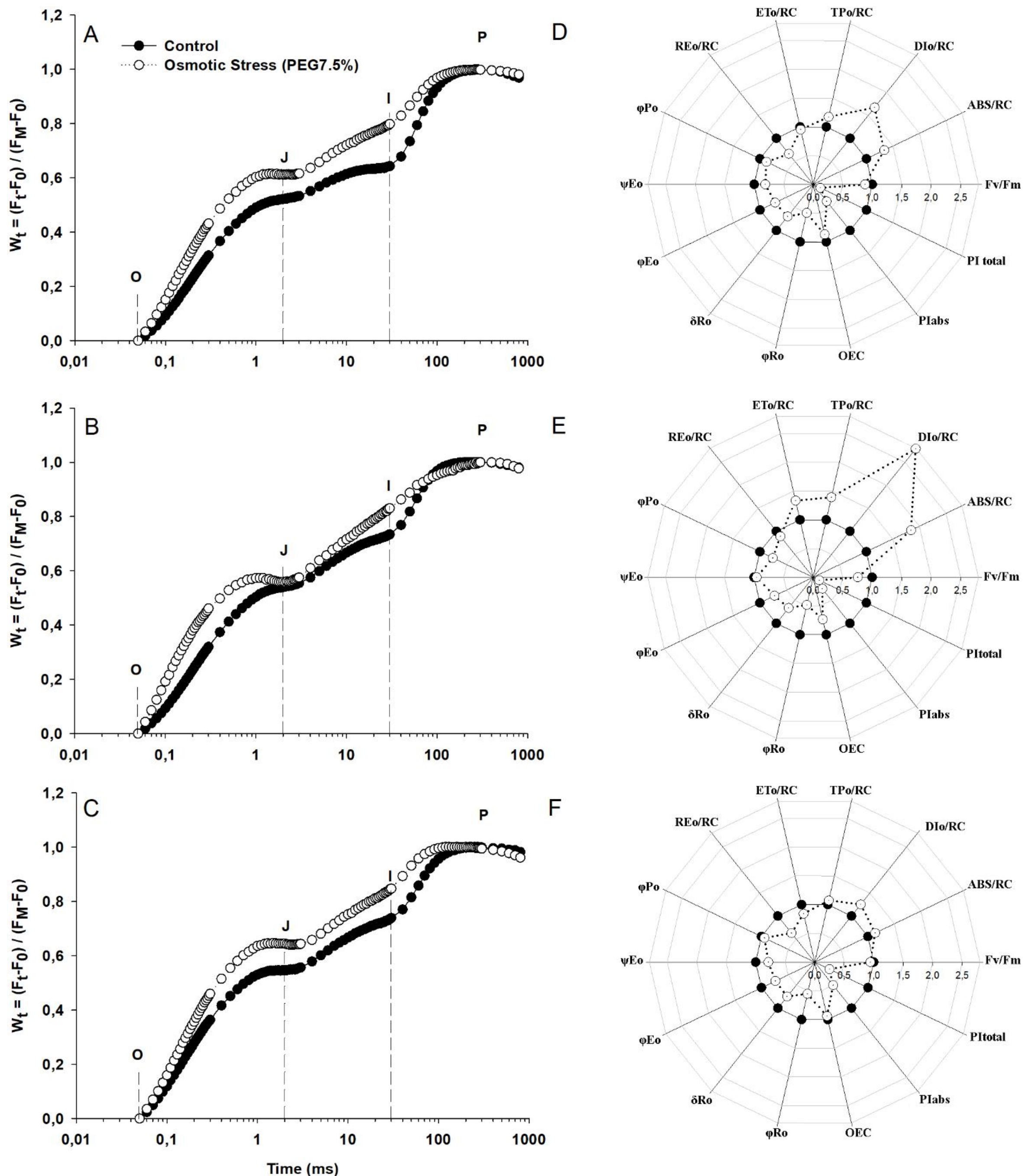


Fig. 4. Chlorophyll *a* fluorescence transients of dark-adapted leaves of *Setaria viridis* grown in hydroponics system without (control – dark circle) and with 7.5% PEG 8000 application (osmotic stress – white circle). Relative variable fluorescence between the steps O and P (W_t) on logarithmic time scale (A-C) and photosynthetic parameters deduced by the JIP-test analysis of fluorescence transients to osmotic stress normalized to control (D-F) at days 3 (A and D), 6 (B and E) and 10 (C and F) after stress imposition. Each curve is the average of 10 replicates. ms milliseconds

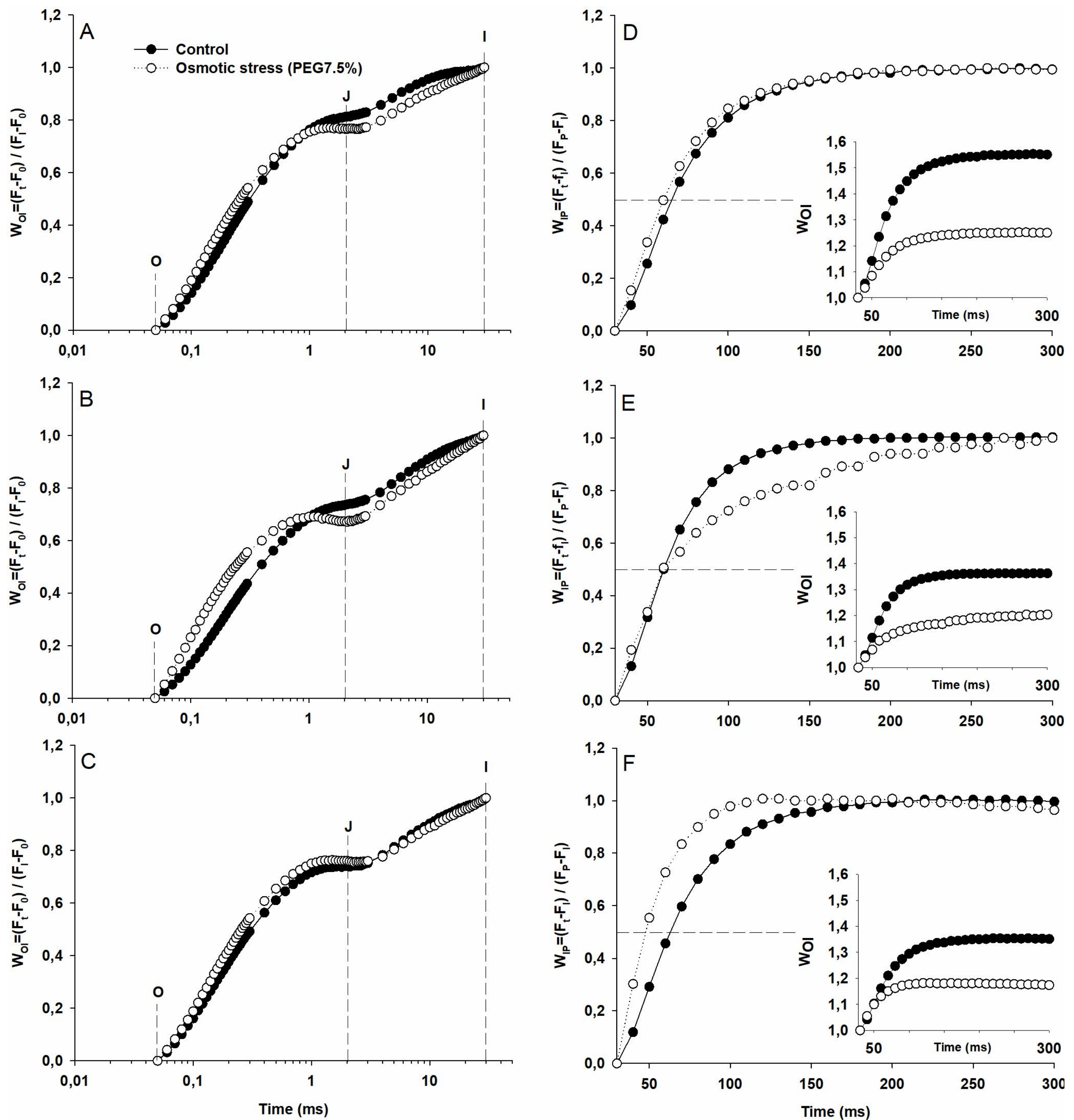


Fig. 5. Chlorophyll *a* fluorescence transients of dark-adapted leaves of *Setaria viridis* grown in hydroponics system without (control – dark circle) and with 7.5% PEG 8000 application (osmotic stress – white circle). Relative variable fluorescence between the steps O and I (W_{OI}; on logarithmic time scale) (A-C) and relative variable fluorescence between the steps I and P (W_{IP}) and W_{OI} in the insert (D-F) at days 3 (A and D), 6 (B and E) and 10 (C and F) after stress imposition. Each curve is the average of 10 replicates. ms milliseconds

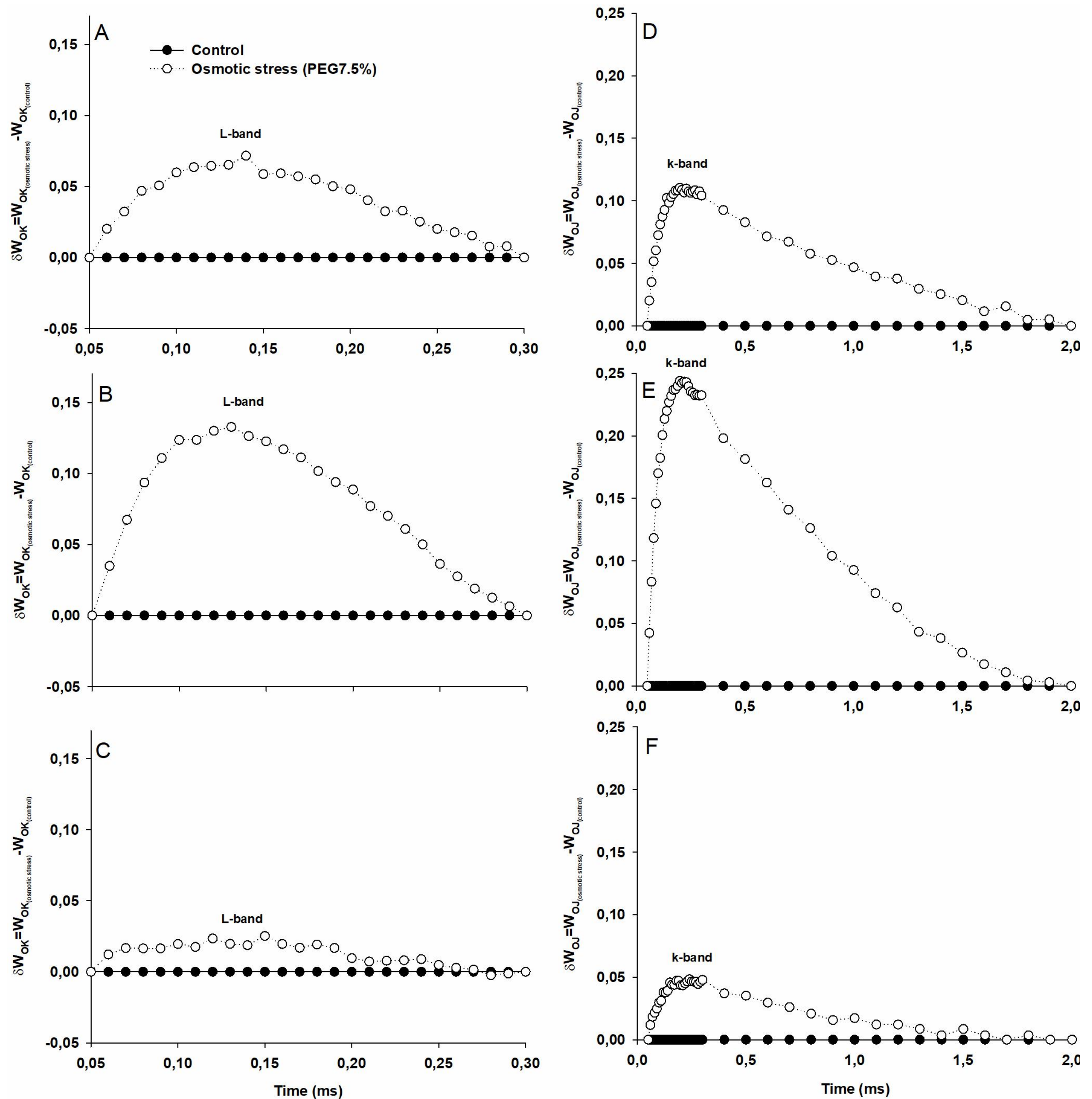


Fig. 6. Chlorophyll *a* fluorescence transients of dark-adapted leaves of *Setaria viridis* grown in hydroponics system without (control – dark circle) and with 7.5% PEG 8000 application (osmotic stress – white circle). Average kinetics depicted between the steps O and K (δW_{OK}), revealing the L-band and average kinetics depicted between the steps O (A-C) and J (ΔW_{OJ}), revealing the K-band (D-F) at days 3 (A and D), 6 (B and E) and 10 (C and F) after stress imposition. Each curve is the average of 10 replicates. ms milliseconds

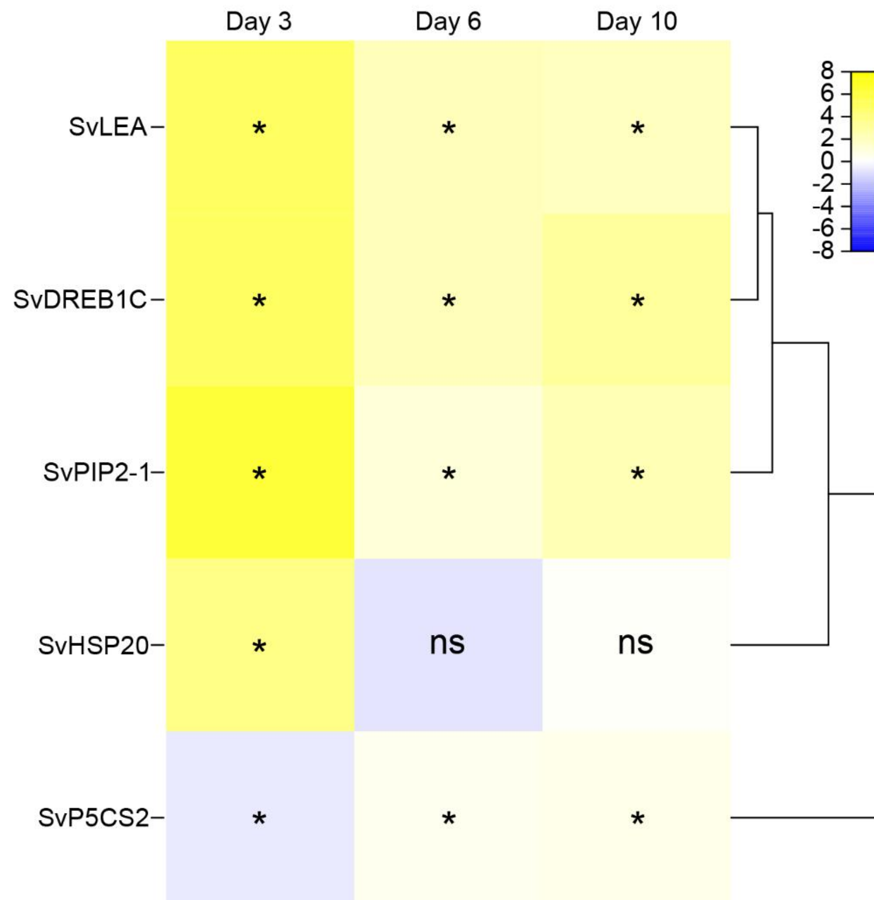
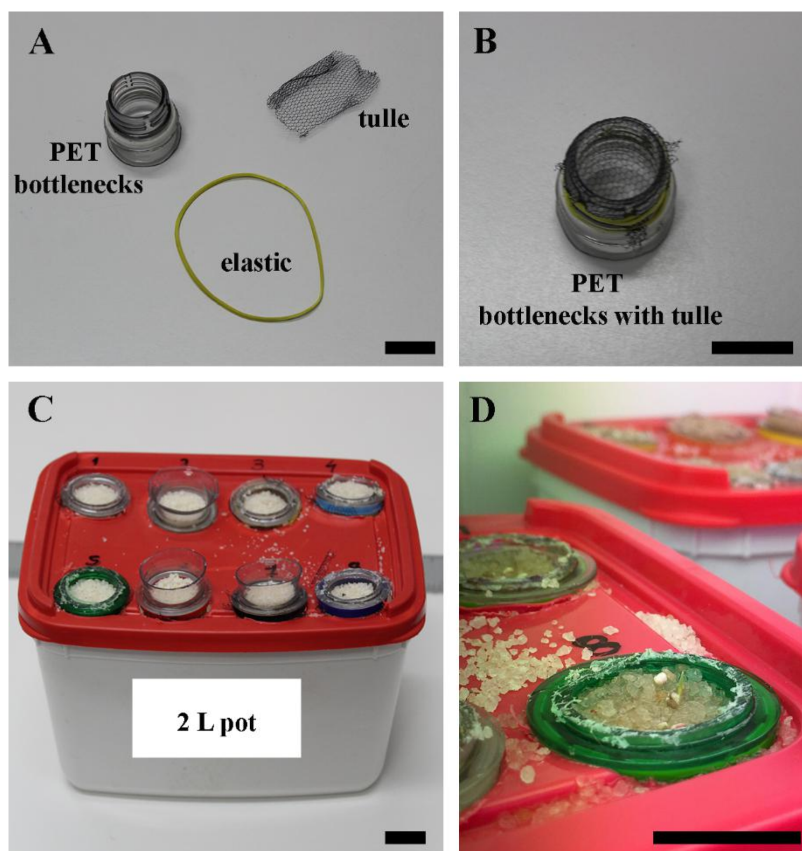
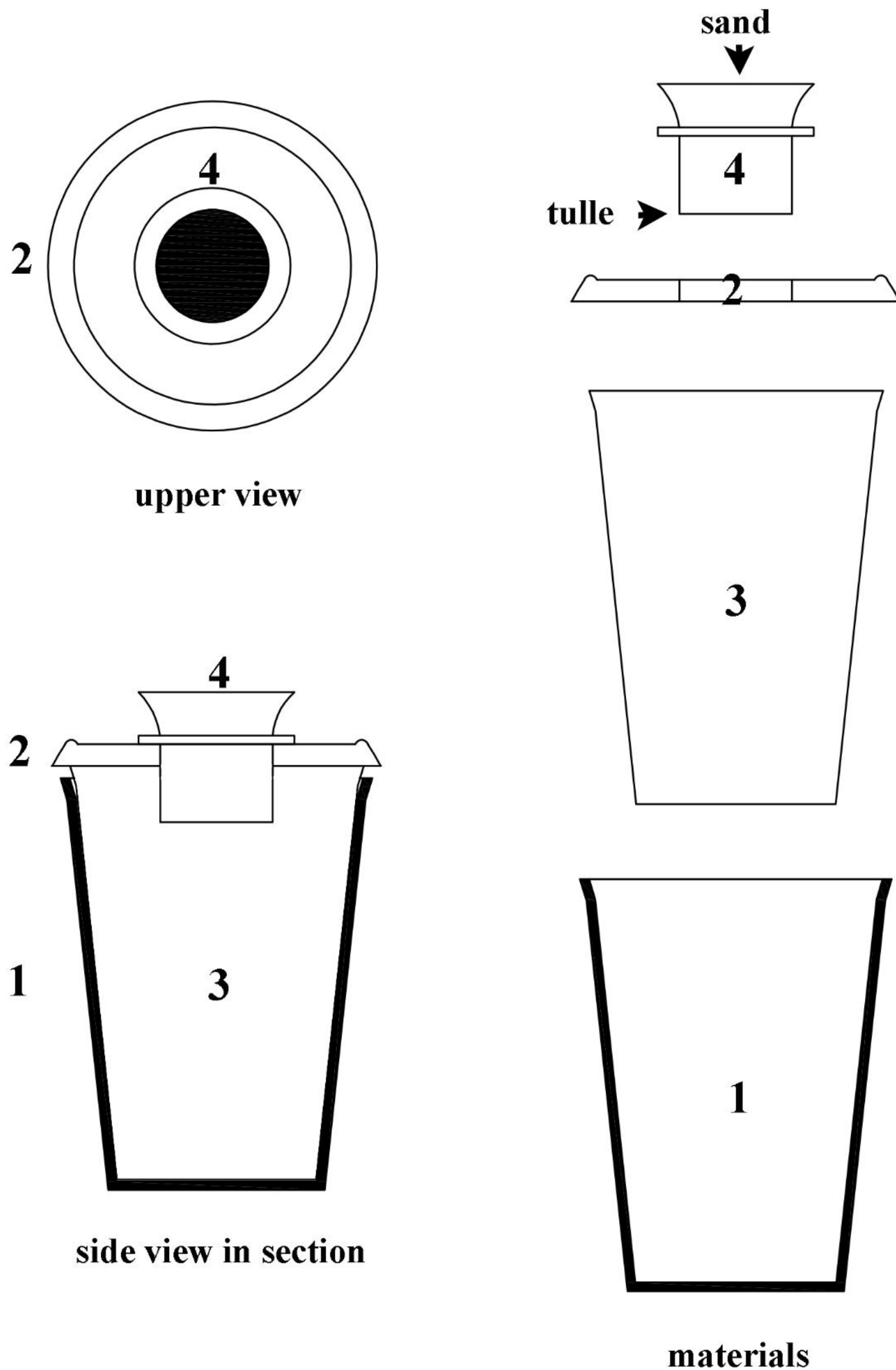


Fig. 7. Expression analysis of the genes *SvLEA*, *SvDREB1C*, *SvPIP2-1*, *SvHSP20* and *SvP5CS2* by qPCR in *Setaria viridis* roots exposed to osmotic stress for 3, 6 and 10 days. The expression data are based on technical triplicate of the three biological replicates (n=3 for replicate) and are presented as Log2FC values relative to treated samples in comparison to the control samples. The significance ($p < 0.05$) of the data was calculated by Relative Expression Software Tool (REST) and the significant results are highlighted with an asterisk while that no significant were indicated with "ns" (not significant). The genes *Seita.1G130900* and *Seita.3G037700* were used as reference genes. The color scale is presented as a yellow spectrum that gradually brightens to the maximal expression value and a blue spectrum to the negative expression values.

Supplementary data



Supplementary Fig. S1. Hydroponics system for seed germination. Consists of a 2 L pot filled with deionized water and eight PET bottlenecks fitted into the lid. Sand was put inside PET bottlenecks and a piece of tulle placed at the bottom of the bottlenecks. Scale Bars: 2 cm.



Supplementary Fig. S2. Hydroponics system to plant growth. Consists of a 0.2 L transparent cup (3), arranged inside a set of 0.2 L dark cup (1) with a dark lid (2) cut in the center for depositing the PET bottlenecks (4). Transparent cups allow visualization of roots and a dark cup prevent light to pass into the nutrient solution and roots. In addition, the enclosure formed by the cup (1) and lid (2) were wrapped with aluminum paper to improve thermal stability of the system. Sand was put inside PET bottlenecks (4) and a piece of tulle placed at the bottom of the bottlenecks.

Supplementary Table S1. Genes evaluated by qPCR. Primer sequences, gene names and gene code based on the *Setaria viridis* and *Setaria italica* genome annotation for each of the genes analyzed by qPCR. The genes *Seita.1G130900* and *Seita.3G037700* were used as reference genes.

Gene code	*Sequence Fw/Rv (5'- 3')*	*Gene name*
Sevir.4G016300	TACGACTACGCGGCAATGTA/ GTCTGCGTCCAATCGTTGC	Dehydration-responsive element binding protein 1C *(SvDREB1C*)
Sevir.5G386300	CAAAATCCATCCGCACTCTT / AATCTGGACCAAGGCATCAG	Delta-1-pyrroline-5-carboxylate synthase 2 (*SvP5CS2*)
Sevir.2G441900	CTCGGAAGCAGCTAGATTGG/ ACCAGTTGTAGGCGATGGAG	Heat shock protein 20 (*SvHSP20*)
Sevir.5G290900	GCCAAGGACAAGGTGGTG/ GCCAGGCTTGTAGGTACTGG	Late Embryogenesis Abundant (*SvLEA*)
Sevir.5G304000	AAGCACGACGAGGTCAAGAT/ ACTCCAGAGACGCAAAAAGC	Aquaporin pip2-1 (*SvPIP2-1*)
Seita.1G130900	CAGTGAGCTATGGAATGGATGA/ GACGCATGTCATGTTGATTCTT	Reference gene 1
Seita.3G037700	TATGGGTCATCAACAGCTTGTC/ GTAGTCCCTCGTGATGAGATCC	Reference gene 2

6. CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Desde que foi proposta como modelo para plantas de metabolismo C_4 por BRUTNEL *et al.*(2010), *S. Viridis* foi sendo muito bem estabelecida, ao longo da última década, como ferramenta para diversos tipos de estudos, incluindo de desenvolvimento, fisiologia e biologia molecular. Pouco se sabe do uso dessa espécie em estudos com herbicidas e a grande maioria desses constam de antes dos anos 2000. Dessa forma, em parceria com o grupo de pesquisa Plantas Daninhas e Pesticidas no Ambiente (PDPA) da UFRRJ, liderado pela professora Camila Ferreira de Pinho, foi possível realizar um estudo avaliando o efeito de cinco herbicidas (paraquat, diuron, paraquate+diuron, clomazone e saflufenacil) que são largamente utilizados.

Parte do trabalho (clomazone e saflufenacil) foi inserido nessa tese de doutorado e o artigo enviado ao periódico South African Journal of Botany foi considerado para publicação após pequenas correções. Outro artigo com os demais herbicidas está em processo de envio ao periódico. A caracterização anatômica e ultraestrutural em avaliações de herbicidas é algo pouco usual, sendo assim, espera-se que esse trabalho torne-se um balizador para futuras pesquisas, tornando o modelo C_4 de *S. viridis* uma ferramenta facilitadora para tal. Ainda, o uso de *S. viridis* no teste de novas formulações de herbicidas poderá também agilizar descobertas nesse campo. Ainda nesse campo, existe uma pesquisa em andamento pelo nosso grupo relacionada ao uso de extrato de *S. viridis* com efeito alelopático na germinação de ervas daninhas.

Baseado nos dados apresentados nesse trabalho pode-se afirmar que o modelo C_4 de *S. viridis* se mostrou promissor como ferramenta biotecnológica em testes com herbicidas e além disso essa gramínea é indicada como muito resistente à estresses abióticos como o déficit hídrico. Por isso, na segunda parte desse estudo, *S. viridis* foi submetida à limitação hídrica por estresse osmótico. Considera-se que existem diversas pesquisas relacionadas ao tema, principalmente nas últimas duas décadas. No entanto entendemos que o que foi desenvolvido aqui nesse estudo traz uma nova visão ao tema, mostrando a recuperação da espécie mantida sob estresse osmótico. Essa recuperação no estresse, foi observada em nossos consecutivos experimentos realizados juntamente com o laboratório de genética molecular vegetal, liderado pelo professor Márcio Alves Ferreira. Estudos anteriores mostravam a recuperação parcial ou completa de *S. viridis* após a reidratação. O nosso grande desafio foi encontrar um sistema de cultivo que nos ajudasse a investigar esse fenômeno. Após diversas abordagens (solo e

hidroponia) chegou-se ao modelo que foi utilizado, desenvolvido pelo nosso laboratório utilizando materiais de baixo custo. Deve-se ressaltar que esse sistema de hidroponia permite o crescimento individual de cada planta, facilitando a observação do crescimento da planta completamente (parte aérea e raiz). Este trabalho foi aceito para publicação, pouco mais de um mês após o envio ao periódico.

Dessa forma, acredita-se que a pesquisa realizada aqui com herbicidas poderá ser continuada afim de estabelecer essa planta como uma ferramenta de avaliação. Similarmente, o estudo realizado com *S. viridis* mantida no estresse osmótico pode ainda ser avaliado em ambiente com CO_2 elevado, o que poderia aumentar a recuperação que foi observada aqui de alguns parâmetros fotossintéticos. Isso pode alavancar pesquisas de melhoramento de outras Panicoideae de interesse comercial, como o arroz, milho e a cana-de-açúcar frente ao cenário atual/ futuro de mudanças climáticas.

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ANEXOS

ANEXO I: Fotografia de parte da primeira página do manuscrito “Changes in Leaf Blade Morphology and Anatomy Caused by Clomazone and Saflufenacil Observed in *Setaria viridis* a Model C_4 Plant” publicado no periódico “South African Journal of Botany” sob o número de DOI:10.1016/j.sajb.2020.09.027.

South African Journal of Botany 135 (2020) 365–376

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Changes in leaf blade morphology and anatomy caused by clomazone and saflufenacil in *Setaria viridis*, a model C_4 plant

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A R T I C L E I N F O

Article History:
Received 12 January 2020
Revised 6 June 2020
Accepted 9 September 2020
Available online xxx

Edited by AR Magee

Keywords:
Foliar surface
C_4 photosynthesis
Ultrastructure
Visible injury
Poaceae

A B S T R A C T

Clomazone and saflufenacil are herbicides extensively used worldwide to weed control. We studied the effects of these two herbicides on morphoanatomical parameters of *Setaria viridis*. Plants were sprayed with four concentrations of each herbicide (clomazone: 500, 1000, 1500 and 2000 g of active ingredient (ai) ha^{-1} and saflufenacil: 49, 98, 147 and 196 g ai ha^{-1}) besides control (without spraying) 20 days after transplantation. The experimental design was completely randomized with five replicates per treatment. Pigment content, visible injuries, morphological and ultrastructural changes were evaluated. No signs of tolerance to either of the tested herbicides were observed. Clomazone caused a decrease in photosynthetic pigment content over time, mostly in young leaves, leading to an “albino” like appearance. There was a reduction in the number of grana in the chloroplasts of mesophyll cells (MC) in necrotic areas. Saflufenacil reduced chlorophyll content, impairing energy absorption in the antenna complex. Injuries to foliar tissues, such as necrosis and depigmentation, were visible as early as 24 h after herbicide application. Bundle sheath chloroplasts (BSC) and MC were completely deformed. The data support the use of *S. viridis* as a model plant for studies on herbicide effects in C_4 monocots.

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ANEXO II: Fotografia de parte da primeira página do manuscrito “Physiological and molecular responses of *Setaria viridis* to osmotic stress” para publicação no periódico “Plant Physiology and Biochemistry” sob o número de DOI:10.1016/j.plaphy.2020.07.019.

Plant Physiology and Biochemistry 155 (2020) 114–125

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Physiological and molecular responses of *Setaria viridis* to osmotic stress

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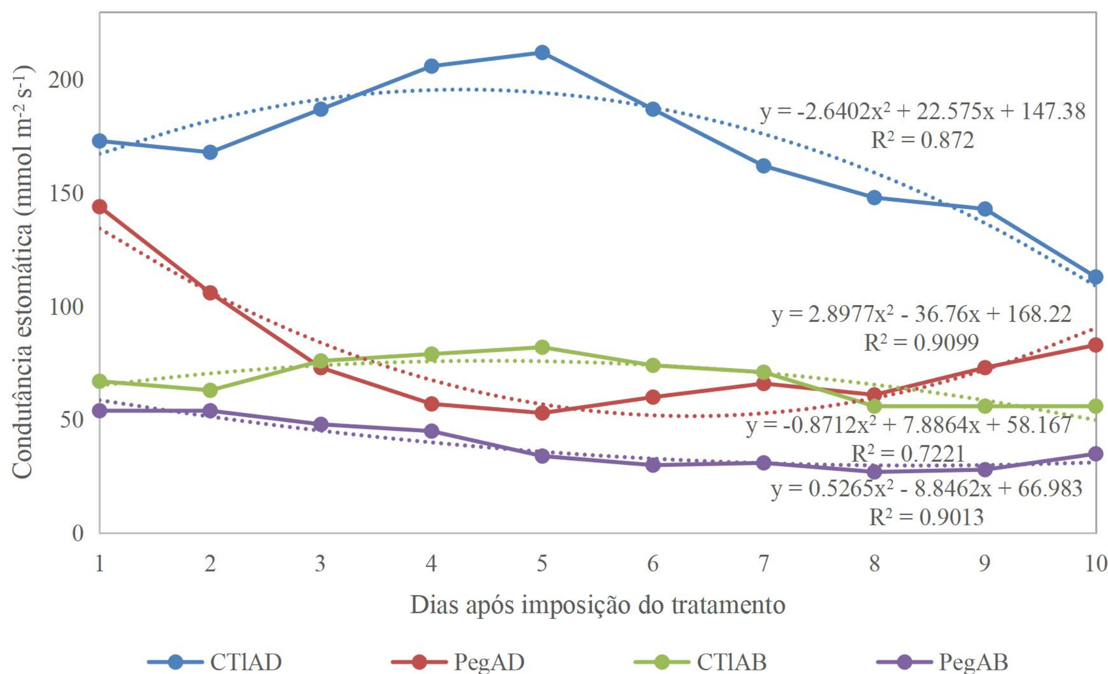
ARTICLE INFO

Keywords:
C_4 model plant
Gene expression analyses
Morphological traits
Photosynthesis
Proline accumulation
Water deficit

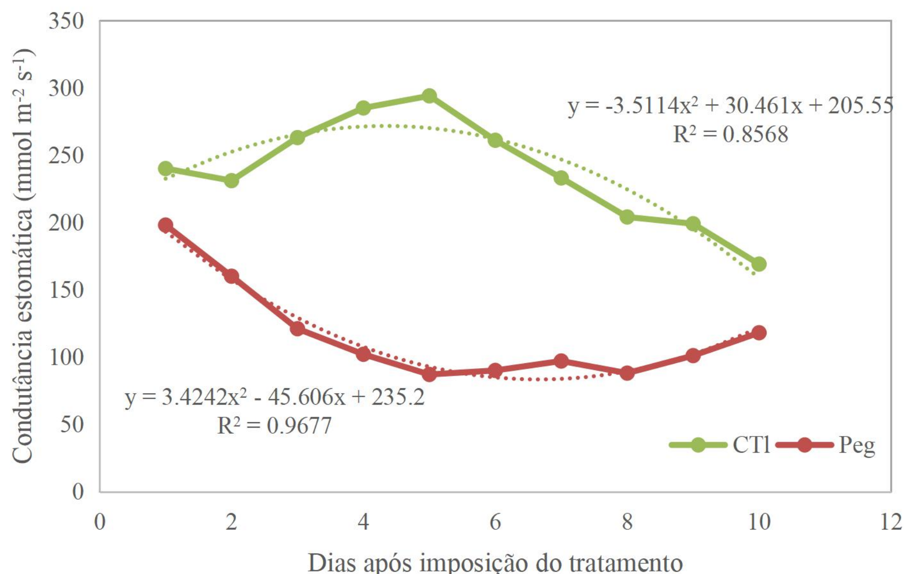
ABSTRACT

Drought-tolerant species, such as *Setaria viridis*, a C_4 model plant, make physiological and biochemical adjustments water limitation and recover from the stress upon its release. We investigated *S. viridis* (A10.1 accession) responses to continuing osmotic stress. The osmotic stress was imposed using polyethylene glycol (PEG) 8000 (7.5%) for 10 days. Morphological traits and stomatal conductance were measured daily for the 10 days. On days 6 and 10, the following traits were measured separately for root and shoot: relative water content (RWC), osmotic potential (OP), electrolytic leakage (EL), and proline content. qPCR analysis was used to evaluate the expression of five selected genes in roots (*SvLEA*, *SvDREB1C*, *SvPIP2-1*, *SvHSP20*, and *SvP5CS2*), and chlorophyll *a* fluorescence was measured on three key days. The morphological data demonstrated a drastic reduction in shoot biomass as an effect of water deficit caused by the osmotic stress. Shoot biomass reduction could be associated with putative ABA-dependent signaling involved in *SvDREB1C* expression. Stomatal conductance and photosynthesis were severely affected up until day 6, however, stomatal conductance and some photosynthetic parameters such as F_V/F_M, ABS/RC, and DI_0/RC showed total or slight recovery on day 10. Root EL decreased in treated plants suggesting an investment in membrane protection by osmoregulator expression such as dehydrin (*SvLEA*) and proline (*SvP5CS2*) genes. Our data suggest that *S. viridis* exhibited a partial recovery from an imposed and constant osmotic stress within 10 days.

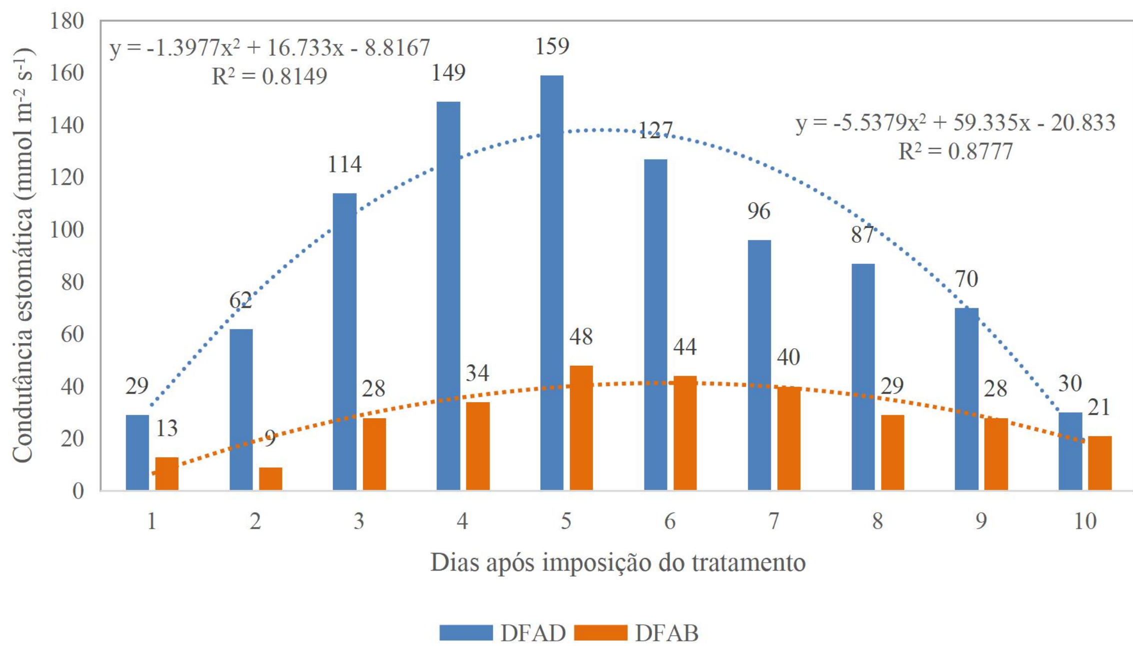
ANEXO III: Representação gráfica da condutância estomática (gs) avaliada em plantas de *Setaria viridis* sob deficit hídrico causado por estresse osmótico. Dados publicados no artigo "Physiological and molecular responses of *Setaria viridis* to osmotic stress" no periódico "Plant Physiology and Biochemistry".



ANEXO Fig A 1: Condutância estomática de plantas controle (CTl) e sob déficit hídrico (Peg) em ambas as faces adaxial (AD) e abaxial (AB) ao longo do experimento.



ANEXO Fig A 2: Somatório das condutâncias estomáticas de ambas as faces, adaxial e abaxial, de plantas controle (CTl) e sob déficit hídrico (Peg) ao longo do experimento.



ANEXO Fig A 3: Diferença entre as condutâncias estomáticas das plantas controle e sob déficit hídrico em ambas as faces, adaxial (DFAD) e abaxial (DFAB), ao longo do experimento.