OXIDATIVE BALANCE AND PHOTOCHEMICAL PERFORMANCE THROUGHOUT THE DEVELOPMENT OF *Sapium glandulosum* (EUPHORBIACEAE) LEAF GALLS INDUCED BY *Neolithus fasciatus* (HEMIPTERA: TRIOZIDAE)

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Orientador: Dr. Vinícius Coelho Kuster
Coorientador: Dr. Diego Ismael Rocha

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CONSERVAÇÃO

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Aos vinte e dois dias do mês de setembro do ano de dois mil e vinte e três, às 09h00min (nove horas), reuniram-se os componentes da banca examinadora em sessão pública realizada por videoconferência, para procederem a avaliação da defesa de Dissertação, em nível de mestrado, de autoria de **MATEUS GOMES THOMÉ,** discente do Programa de Pós-Graduação em Biodiversidade e Conservação do Instituto Federal Goiano – Campus Rio Verde. A sessão foi aberta pelo presidente da Banca Examinadora, Prof. Dr. Vinícius Coelho Kuster, que fez a apresentação formal dos membros da Banca. A palavra, a seguir, foi concedida ao autor para, em 30 min., proceder à apresentação de seu trabalho. Terminada a apresentação, cada membro da banca arguiu o examinado, tendo-se adotado o sistema de diálogo sequencial. Terminada a fase de arguição, procedeu-se a avaliação da defesa. Tendo-se em vista as normas que regulamentam o Programa de Pós-Graduação Biodiversidade e Conservação, e procedidas às correções recomendadas, a Dissertação foi APROVADA, considerando-se integralmente cumprido este requisito para fins de obtenção do título de **MESTRE EM BIODIVERSIDADE E CONSERVAÇÃO,** na área de concentração em Conservação dos Recursos Naturais, pelo Instituto Federal Goiano – Campus Rio Verde. A conclusão do curso dar-se-á quando da entrega na secretaria do PPGBio da versão definitiva da Dissertação, com as devidas correções. Assim sendo, a defesa perderá a validade se não cumprida essa condição, em até **60 (sessenta) dias** da sua ocorrência. A Banca Examinadora recomendou a publicação dos artigos científicos oriundos dessa dissertação em periódicos após procedida as modificações sugeridas. Cumpridas as formalidades da pauta, a presidência da mesa encerrou esta sessão de defesa de Dissertação de Mestrado, e para constar, foi lavrada a presente Ata, que, após lida e achada conforme, será assinada eletronicamente pelos membros da Banca Examinadora.

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BIOGRAFIA DO AUTOR

Mateus Gomes Thomé, filho de Michele Cristina Gomes e Ronaldo Rodrigues Thomé. Nascido em Penápolis, SP, em 26 de agosto de 1997.

Concluiu o ensino médio na Escola Estadual São José, MS, em 2014. Iniciou no curso de Ciências Biológicas, Licenciatura, pela Universidade Federal de Jataí em 2016, com conclusão no segundo semestre de 2021. Ingressou no Programa de Pós-graduação em Biodiversidade e Conservação no Instituto Federal Goiano, campus Rio Verde, GO, em setembro de 2021.

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Sigla	Significado	Unidade
APX	Ascorbate peroxidase	nmol min^{-1} mg^{-1} of protein
AsA	Ascorbic acid	
CAT	Catalase	nmol min^{-1} mg^{-1} of protein
Cu	Copper	
DAB	3,3' diaminobenzidine	
DM	Dry mass	
F_0	Minimum fluorescence in the state adapted to the dark	
Fe	Iron	
F_M	Maximum fluorescence in the state adapted to the dark	
FM	Fresh mass	
$(F'_m-F')/F'_m$	PSII operational efficiency	
F_v/F_m	Maximum quantum yield	
FW	Fresh weigth	
H_2O_2	Hydrogen peroxide	nmol g^{-1} FW
MDA	Malondialdehyde	nmol g^{-1} FW
Mg	Milligrams	
Min	Minutes	
Mn	Manganese	
mmol	Milimoles	
NPQ	Non-photochemical quenching during light adaptation	
$^1O_2$	Singlet oxygen	
O_2	Molecular oxygen	
O_2^-	Superoxide anion	nmol g^{-1} FW
OH^-	Hydroxyl radical	
POX	Peroxidase	nmol min^{-1} mg^{-1} of protein
Prot	Protein	
PSII	Photosystem II	
RWC	Relative water content	
ROS	Reactive oxygen species	
R_{fd}	Steady-state fluorescence decline ratio	
SOD	Superoxide dismutase	nmol min^{-1} mg^{-1} of protein
TM	Turgid mass	
TSPC	Total soluble phenolic compounds	mg/kg
Zn	Zinc	

RESUMO

THOMÉ, MATEUS GOMES. Instituto Federal Goiano, câmpus Rio Verde, GO, setembro de 2023. **Oxidative balance and photochemical performance throughout the development of *Sapium glandulosum* (Euphorbiaceae) leaf galls induced by *Neolithus fasciatus* (Hemiptera: Triozidae)** Orientador: Vinícius Coelho Kuster. Coorientador: Diego Ismael Rocha. Programa de Pós-Graduação em Biodiversidade e Conservação

A indução de galhas nas plantas hospedeiras geralmente é associada a um aumento do estresse oxidativo mediado pelas espécies reativas de oxigênio. O estresse é regulado por vias de dissipação que minimizam a peroxidação lipídica no tecido galhado. Assim, o objetivo desse estudo foi avaliar os níveis de estresse oxidativo, suas vias de dissipação e o impacto no metabolismo fotossintético durante o desenvolvimento das galhas foliares de *Sapium glandulosum* (Euphorbiaceae) induzidas por *Neolithus fasciatus* (Hemiptera: Triozidae). Para isso, folhas não-galhadas e galhas nos estágios jovem, maduro e senescente foram submetidas a análises histoquímicas, da atividade fotossintética, dosagem de pigmentos, quantificação do estresse oxidativo, substâncias fenólicas e de enzimas antioxidantes. O peróxido de hidrogênio foi marcado no tecido fotossintético da folha e nos feixes vasculares das galhas durante os diferentes estágios de desenvolvimento. O conteúdo de malondialdeído foi maior nas folhas e nas galhas senescentes, já a quantificação de fenólicos não apresentou diferença entre a folha e as galhas. A atividade da catalase, peroxidase e ascorbato peroxidase foram superiores nas folhas com relação às galhas. Diferentemente, para superóxido dismutase, os maiores valores foram encontrados para as galhas em relação à folha. Os teores de clorofila chegaram a ser quatorze vezes maiores na folha do que nos três estágios de desenvolvimento das galhas. Mesmo assim, não houve diferença no rendimento quântico máximo entre os tratamentos exceto para galhas senescentes. A galha de *S. glandulosum* parece regular eficientemente o estresse oxidativo principalmente pela atividade do superóxido dismutase, mantendo seu rendimento fotoquímico similar ao da folha.

Palavras-chave: Enzimas antioxidantes; Estresse oxidativo; Fotossíntese; ROS.

ABSTRACT

THOMÉ, MATEUS GOMES. Instituto Federal Goiano, câmpus Rio Verde, GO, setembro de 2023. **Oxidative balance and photochemical performance throughout the development of *Sapium glandulosum* (Euphorbiaceae) leaf galls induced by *Neolithus fasciatus* (Hemiptera: Triozidae)** Supervisor: Vinícius Coelho Kuster. Co-supervisor: Diego Ismael Rocha. Postgraduate program – Master in 'Biodiversidade e Conservação'

The induction of galls in the host plants is usually associated with an increase in oxidative stress mediated by reactive oxygen species. The stress is regulated by dissipation pathways, which minimize lipid peroxidation in the galled tissues. Thus, the aim the current study was to evaluate the levels of oxidative stress, its dissipation pathways, and the impact on photosynthetic metabolism during the development of leaf galls of *Sapium glandulosum* (Euphorbiaceae) induced by *Neolithus fasciatus* (Hemiptera: Triozidae). So, non-galled leaves and galls in the young, mature, and senescent stages were submitted to histochemical analysis, photosynthetic activity, pigment dosage, quantification of oxidative stress, phenolic substances and antioxidant enzymes. Hydrogen peroxide was labeled in the photosynthetic tissue of the leaf and in the vascular bundles of the galls during the different stages of development. The malondialdehyde content was higher in the leaves and senescent galls, while the quantification of phenolics showed no difference between the leaf and galls. The activity of catalase, peroxidase and ascorbate peroxidase were higher in the leaf than galls. In contrast for superoxide dismutase, the higher values for galls were found. The chlorophyll contents were fourteen times higher in the leaf than in the three developmental stages of galls, yet there was no difference in the maximum quantum yield between the treatments except for senescent galls. The gall of *S. glandulosum* seems to efficiently regulate oxidative stress mainly by the activity of superoxide dismutase, maintaining its photochemical yield similar to that of the leaf.

Keywords: Antioxidant enzymes; Oxidative stress; Photosynthesis; ROS.

1. INTRODUCTION

Galls are neoformed organs in host plants developed after the induction of different organisms (Mani 1964; Shorthouse *et al.* 2005). The gall formation changes in the morphogenic patterns of the host plant, structuring an environment that provides for the gall-inducer shelter, nutrition, and protection against natural enemies and abiotic factors (Ramalho 2010). Insect-induced galls are usually the most complex, with specialized tissues affected by galler feeding habits that can influence the level of oxidative stress (Ferreira *et al.* 2019; Kuster *et al.* 2022), usually higher in galls than the non-galled organs (Oliveira *et al.* 2006; Moura *et al.* 2008; Oliveira & Isaias 2009; Oliveira *et al.* 2016).

The main stress molecule are reactive oxygen species (ROS), produced naturally by the intrinsic plant metabolism in mitochondria and chloroplasts (Harir *et al.* 2009) as well as over the course of gall development under biotic stress (Isaias *et al.* 2015). Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$) are examples of ROS, being formed by the reduction of the molecular oxygen (O_2) (Apel & Hirt 2003). These molecules are signalers of plant development (Apel & Hirt 2003; Isaias *et al.* 2015), but at high levels they can be harmful to cells, providing damages in the lipid membranes and in the genetic material (Jaleel *et al.* 2009). The inefficiency in the control of oxidative stress can cause a homeostatic imbalance and a consequent peroxidation of membrane lipids, with the formation of malondialdehyde (MDA) (Bailly *et al.* 2000).

Reduction in photosynthetic rates in galls has been associated with increased oxidative stress in their tissues (Oliveira *et al.* 2011; Castro *et al.* 2012; Isaias *et al.* 2015), as demonstrated for galls induced by *Eugeniamyia dispar* (Cecidomyiidae) on

the leaves of *Eugenia uniflora* (Myrtaceae) (Rezende *et al*. 2018). The reduction of photosynthetic capacity in galls can be related to the lower content of chloroplastid pigments and the expressive cellular hypertrophy (Oliveira *et al*. 2017), as well as to the redifferentiation of photosynthetic tissue from the non-galled organ to new functional compartments, the gall (Ferreira *et al*. 2019). Maintaining photosynthetic machinery in the gall can reduce hypoxia levels and hypercarbia (Haiden *et al*. 2012; Oliveira *et al*. 2017), which are usually high in galls because they have compact tissues and few or no stomata (Heldt & Piechulla 2010). Thus, photosynthetic activity may be multi-usefull for galls. In some cases, they may maintain the same levels that the non-galled organs. (Oliveira *et al*. 2011), even as, in rare cases, the levels may be like that of the non-galled organs (Fernandes *et al*. 2010; Oliveira *et al*. 2011).

The production of phenolic compounds acts on the uptake and dissipation of ROS in galls, contributing to their homeostatic balance (Isaias *et al*. 2015; Smith 2002). Associated with these compounds, an efficient enzymatic antioxidant defense system, i.e., peroxidase (POX), ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD), also aids in the control of oxidative homeostasis acting in the scavenging of ROS in plant tissues (Posmyk *et al*. 2009; Yang *et al*. 2008; Zagorchev *et al*. 2018). The role of phenolic compounds in the dissipation of ROS in galls has been discussed in Neotropical host-gall systems (Ferreira *et al*. 2018; Kuster *et al*. 2019), but there are few studies approaching antioxidant enzymes essays, which affects the real understanding of stress dissipation pathways in galls.

The POX is an oxidoreductase found mainly in cell walls and vacuoles, reducing H_2O_2 stress acting as an oxidant (Barbosa *et al*. 2014). POX is involved in the process of lignification of the cell wall, tissue healing, plant development, and defense against pathogens, among other functions (Gaspar *et al*. 1982). APX is a class I hemeproteins of

the peroxidase superfamily, which can be found in the cytosol, mitochondria, peroxisomes, chloroplasts, and cell walls (Gill & Tuteja 2010; Mittler 2002). It acts in the elimination of H_2O_2 using ascorbic acid (AsA) as a reducer (Barbosa *et al*. 2014; Gill & Tuteja 2010). CAT acts on peroxisomes, glyoxysomes and mitochondria, in a process of ROS detoxification mainly under stress conditions, as it catalyzes the decomposition of H_2O_2 to H_2O and O_2 during photorespiration (Gill & Tuteja 2010; Barbosa *et al*. 2014). SOD is a metallo-enzyme that provides the first line of defense against ROS by catalyzing the dismutation of two superoxide anions O_2^{-} into H_2O_2 and O_2 in chloroplasts, mitochondria, cytosol, and peroxisomes (Mittler 2002). In general, they are found in different sites in the plant body, e.g., Mn-SOD in the mitochondrial matrix, cytosolic Cu/Zn-SOD and Fe-SOD present in the chloroplast stroma, and their amount varied from plant to plant (Bowler *et al*. 1992).

The aim of the present study was to evaluate the levels of oxidative stress and its dissipation pathways during the development of leaf galls of *Sapium glandulosum* (L.) Morong (Euphorbiaceae) induced by *Neolithus fasciatus* Scott, 1882 (Hemiptera: Triozidae), as well as the influence of gall formation and development in photosynthetic activity. The high number of galls on *S. glandulosum* allows biochemical analyses, which were the main reason for choosing that system. The following questions were addressed: (i) What are the levels of oxidative stress in galls? (ii) How is the antioxidant system in galls composed? and (iii) What is the impact of gall stress on the quantum yield of photosynthesis and other associated parameters?

2. MATERIAL AND METHODS

2.1 Plant material and study area

Sapium glandulosum (L.) Morong (Euphorbiaceae) (Figure 1A) is popularly known as a "leiteira" and occurs in the Jataí municipality, Goiás state (17° 52′ 33" S, 51° 43′ 17" W), Brazil. *Sapium glandulosum* has large green globoid leaf galls induced by *Neolithus fasciatus* Scott, 1882 (Hemiptera: Triozidae). The galls are completely closed and the individual chambers open when the gall inducers reach the adult stage (Hanson *et al*. 2014). The leaves of *S. glandulosum* have a uniseriate epidermis and are hypostomatic, with dorsiventral mesophyll and collateral vascular bundles (Rosa 2022). The young gall has a uniseriate epidermis, with few stomata and cortex composed of parenchyma with reduced cells and collateral vascular bundles (Rosa 2022). Mature galls have larger cortex cells with starch grains, as well as hypertrophied vascular bundles (Rosa 2022). Senescent galls have a histological structure like the mature stage, but with some collapsed epidermal cells and cortex with apparently absent cellular content (Rosa 2022).

For the present study, mature non-galled leaves of the 5th node (n= 3-10) (Figure 1B) and galls (n= 3-10) were used for physiological, histochemical, biochemical and stress analyses, with evaluation of galls in three stages of development: young i.e., growing and developing (Figure 1C), mature (Figure 1D) and senescent (Figure 1E). Young galls were small and in growing, with gall inducer on the 2nd or 3rd instar. Mature galls had ended differentiation, with inductor in the 5th instar. Senescent galls no longer possessed the gall inducer, with an open ostiole in its apical portion.
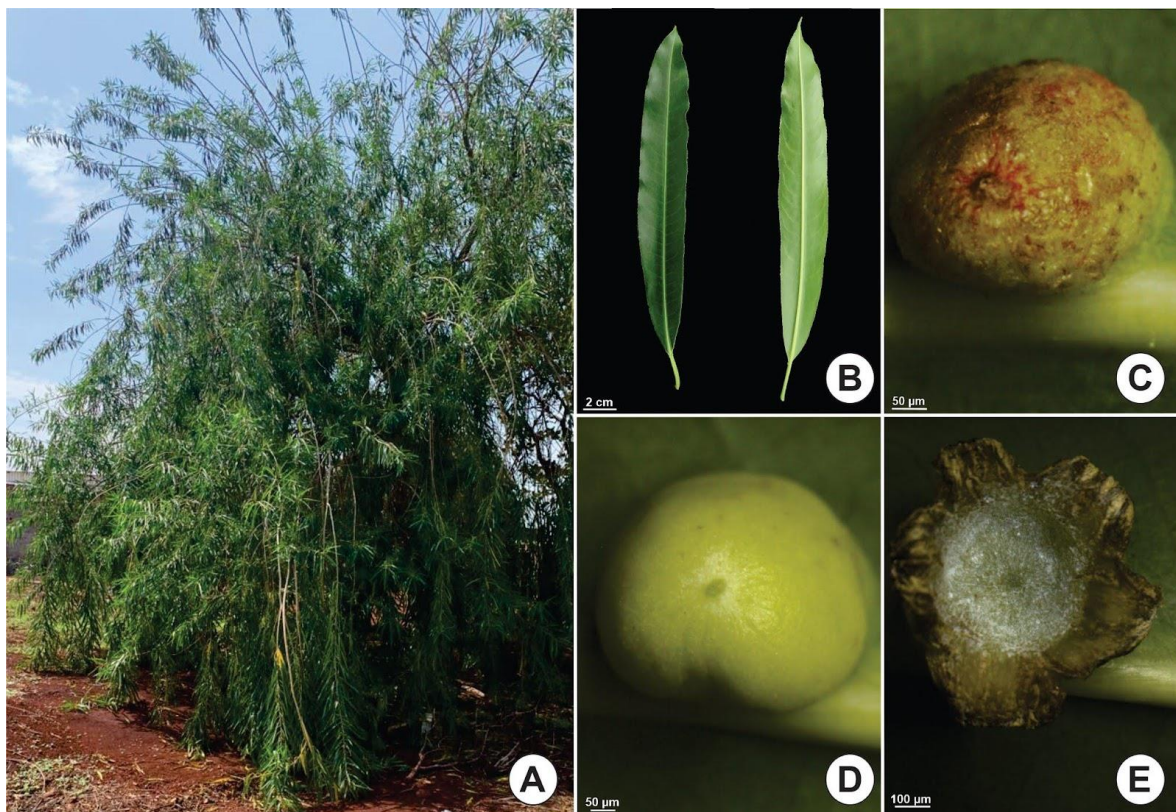
Figure 1. Individual of the host plant, *Sapium glandulosum* in the field (A), leaves (B) and galls induced by *Neolithus fasciatus*, in three stages of development: young (C), mature (D) and senescent (E).

2.2 Relative water content (RWC)

The relative water content was measured in leaf discs of 1cm^2 of the middle portion of the leaf (n=10) and gall, in the different stages of development (n=10). The collection was carried out between 8:00 and 9:00 am, with subsequent weighing on an analytical balance (Marte® AL 500C) to obtain the fresh mass (FM). Then, the samples were immersed in water for 48 h and weighed to obtain the turgid mass (TM), followed by drying in an oven for 48 h at 50 ℃ to obtain the dry mass (DM). From these data, the RWC was obtained according to the calculation of Turner (1981), where the RWC = ((FM-DM)/(TM-DM)) X 100.

2.3 H_2O_2 histolocalization

The histochemical analyses were based on fresh plant samples, and the sections were freehand made. For the labeling of hydrogen peroxide, leaf and gall samples at different stages of development (n= 3) were submitted to 3,3' diaminobenzidine (DAB) for 20 minutes in the dark (Rosseti & Bonnatti 2001). Then, the samples were washed and mounted in distilled water. All slides were photographed under a light microscope (Leica DM750) with a digital camera attached (Leica ICC50 HD).

2.4 Oxidative stress analyses

For the quantification of oxidative stressful and ROS stress-dissipating molecules, samples of leaves (n=6) and galls in the three stages of development (n=6 for each stage) were collected in the field and immediately inserted into liquid nitrogen, with subsequent storage in an ultrafreezer at -80°C until analyses.

The quantification of hydrogen peroxide (H_2O_2) was performed in 0.100 g of samples, which were macerated in liquid nitrogen, homogenized in potassium phosphate buffer 50 mM (pH 6.5) and hydroxylamine 1mM, and then centrifuged at 10.000 xg for 15 min at 4 °C (Kuo & Kao 2003). The supernatant was added to the reaction medium consisting of 100 μM FeNH4(SO4), 25 mM sulfuric acid, 250 μM xylenol orange and 100 mM sorbitol (Gay & Gerbicki 2000). The samples were kept in the dark for 30 min and the absorbance was determined at 560 nm. The H_2O_2 concentration was estimated based on a standard H_2O_2 curve and was expressed in nmol g^{-1} of fresh weight (FW).

The concentration of superoxide anion $O_2^{.-}$ was determined in 0.100 g of samples, homogenized in sodium phosphate buffer 100 mM (pH 7.0), containing 1 mM

of sodium diethyldithiocarbamate (DDC). The homogenate was centrifuged at 22.000 xg for 20 min at 4 °C. After centrifugation, 0.1 ml of the supernatant was added to 1.9 ml of the solution consisting of sodium phosphate buffer 100 mM (pH 7.0), DDC 1 mM of p-nitrotetrazolium blue 0.25 mM. The concentration of O_2^- was determined by the absorbance of the product subtracted from the initial absorbance at 540 nm (ABS 540 $\text{min}^{-1}\ \text{g}^{-1}$FW) (Chaitanya & Naithani 1994).

The quantification of Malondialdehyde (MDA), which reflects lipid peroxidation in tissues, was based on the protocol described by Cakmak & Horst (1991). Plant samples of 0.150 g were macerated in liquid nitrogen, and the material was subsequently homogenized in 2 mL of 1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 15.000 xg for 20 min at 4 °C. After centrifugation, 0.5 mL of the supernatant was added to 1.5 mL of 0.5% thiobarbiuric acid solution (m/v) and incubated in a water bath at 95 °C for 30 min. After that, the reaction was stopped in an ice bath and the samples were centrifuged at 9.000 xg for 5 min at 15 °C. The MDA concentration was calculated using the extinction coefficient of 155 $\text{mM}^{-1}\ \text{cm}^{-1}$ and expressed in nmol g^{-1} of fresh weight (FW) (Heath & Packer 1968).

2.5 Analyses of the antioxidant system that dissipate oxidative stress

At first, 0.250 g of samples were macerated in liquid nitrogen, and homogenized in 2 mL of potassium phosphate buffer 50 mM (pH 6.8), containing ethylenediaminetetraacetic acid (EDTA) 0.1 mM, phenylmethylsulfonic fluoride (PMSF) 1 mM and polyvinylpyrrolidone (PVPP) 5 % (m/v). The homogenate was centrifuged at 15.000 xg for 20 min at 4 °C and the supernatant was used as an extract for enzymatic determinations. After that, enzymatic extract was used to determine the activity of the enzymes catalase (CAT), ascorbate peroxidase (APX), superoxide

dismutase (SOD), and peroxidase (POX). The protein concentration in each sample was determined by the Bradford method (1976) to obtain the standard curve.

The CAT activity was determined by the method of Cakmak & Marshner (1992). The reaction mixture consisted of 50 Mm potassium phosphate buffer (Ph 7.0) and H_2O_2 in a volume of 2 mL. The reaction was initiated by the addition of 50 μL of the sample extracts, in which the activity was determined by the consumption of H_2O_2 at 240 nm for 1 min at 25 °C. The molar extinction coefficient of 36 M^{-1} cm (Anderson *et al.* 1995) was used to determine the activity of CAT, which was expressed in nmol min^{-1} mg^{-1} of protein.

The APX activity was determined by the method of Nakano & Asada (1981), with some modifications. 50 μL of the samples were added to a solution composed of potassium phosphate buffer 50 mM (pH 6.8), H_2O_2 1 mM and ascorbate 0.8 mM, and the final volume was corrected to 2 mL. The reaction was measured by the oxidation of ascorbate dependent H_2O_2 at 290 nM for 5 min at 25ºC. The molar extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate the APX activity, which was expressed in μmol min^{-1} mg^{-1} of protein.

The SOD activity was determined by adding 50 μL of the samples to 1.94 mL of reaction mixture consisting of distilled water 50 μL, sodium phosphate buffer 50 nM (Ph 7.8), methionine 13 nM, p-nitrotetrazolium blue (NBT) 75 μM, EDTA 0.1 nM and riboflavin 2 μM (Del Longo *et al.* 1993). The reaction occurred at 25 °C under illumination of 15 W lamps. After 5 min of light exposure, the illumination was interrupted and the blue formazan produced by NBT photoreduction was measured in a spectrophotometer (Evolution 60, Thermo Fisher Scientific Inc., Massachusetts - USA), at 560 nM (Giannopolittis & Ries 1977). The control samples had their absorbances

measured at 560 nM, using reaction mixture kept in the dark for 5 min. The values obtained were subtracted from the sample readings of the repetitions of the treatments that received illumination. SOD activity was expressed in SOD units per min^{-1} mg^{-1} of protein.

The POX activity was determined by the methodology proposed by Kar & Misha (1976), by the oxidation of pyrogallol. The reaction mixture consisted of distilled water, potassium phosphate buffer 25 mM (pH 6.8), pyrogallol 20 nM and H_2O_2 nM in a volume of 2 mL. The reaction was initiated by the addition of 50 μL of the sample, and the activity was determined by the consumption of H_2O_2 at 420 nM, for 1 min, at 25 °C (Chance & Maehley 1955). POX activity was expressed in μmol of purpurogalin produced min^{-1} mg^{-1} of protein.

For total soluble phenolic compounds (TSPC), 0.1 g of samples were macerated with liquid nitrogen with subsequent addition of 1 mL of 80% methanol. The solution remained for 4 h on an agitator table at 300 rpm at room temperature and, subsequently, was centrifuged at 17.000 xg for 30 min. The supernatant was used for the determination of TSPC. The concentration of TSPC was determined through the method developed by Zieslin & Ben Zaken (1993), with some modifications. The reaction was initiated with the addition of 0.2 M Folin-Ciocalteu Phenol at 150 μL of the samples, followed by incubation at 25ºC for 5 min and addition of 0.1 M sodium carbonate. Absorbance was obtained at 725 nm and TSPC concentration was performed based on a calibration curve using gallic acid as standard.

2.6 *Determination of photosynthetic activity and cloroplastidic pigments*

The measurement of chlorophyll *a* fluorescence (n= 8) was made directly in the field using a Handy FluorCam – PSI portable equipment (Photon Systems Instruments,

Czech Republic). Measurements were obtained between 8.00 and 10.00 am after adaptation to the dark for 30 minutes (Rascher *et al*. 2000) on leaves and galls in the three stages of development. The following parameters were obtained: F_0 = minimum fluorescence in dark-adapted state; F_M= maximum fluorescence in dark-adapted state; Fv/Fm = maximum PSII quantum yield in dark-adapted state; NPQ = steady-state non-photochemical quenching; R_{fd} = fluorescence decline ratio in steady-state and (F'_m-F')/F'_m = PSII operational efficiency (where F'm is the fluorescence signal when all PSII centers are closed in the light-adapted state, and F' is the measurement of the light-adapted fluorescence signal).

Photosynthetic pigments (n= 8) were quantified using leaf discs of 1 cm^2, immersed in 80% acetone for 48 hours and centrifuged at 1.500 rpm for 5 minutes in a centrifuge (Fanen Mod 206 BL). The absorbance of the supernatant at 470, 646 and 663 nm was performed in a spectrophotometer (Thermo Spectronic Mod. Genesys 10 UV) and the calculation of chlorophyll *a*, chlorophyll *b* and carotenoids performed according to Lichtenthaler & Wellburn (1983).

2.7 Statistical analyses

The quantitative data were initially submitted to the normality and homoscedasticity test. Thus, ANOVA was used, followed by Tukey test in Rstudio® for all analyses. 5% significance was adopted.

3. RESULTS

3.1 Histochemical analyses

Hydrogen peroxide was detected in leaves, mainly in the chlorophyllous tissue and in the vascular bundles (Figure 2A). This compound was present in the different

stages of gall development, being detected in the epidermis, chlorophyllous outer cortex and vascular bundles of young galls (Figure 2B, C). In the mature galls, hydrogen peroxide was histolocalized mainly in the cortex that surrounds the nymphal chamber and in the vascular bundles (Figure 2D, E), while in the senescent galls the labeling was concentrated in the epidermis, vascular bundles, and cortex (Figure 2F). The laticifers showed extensive hydrogen peroxide labeling throughout the development of the galls (Figure 2G).
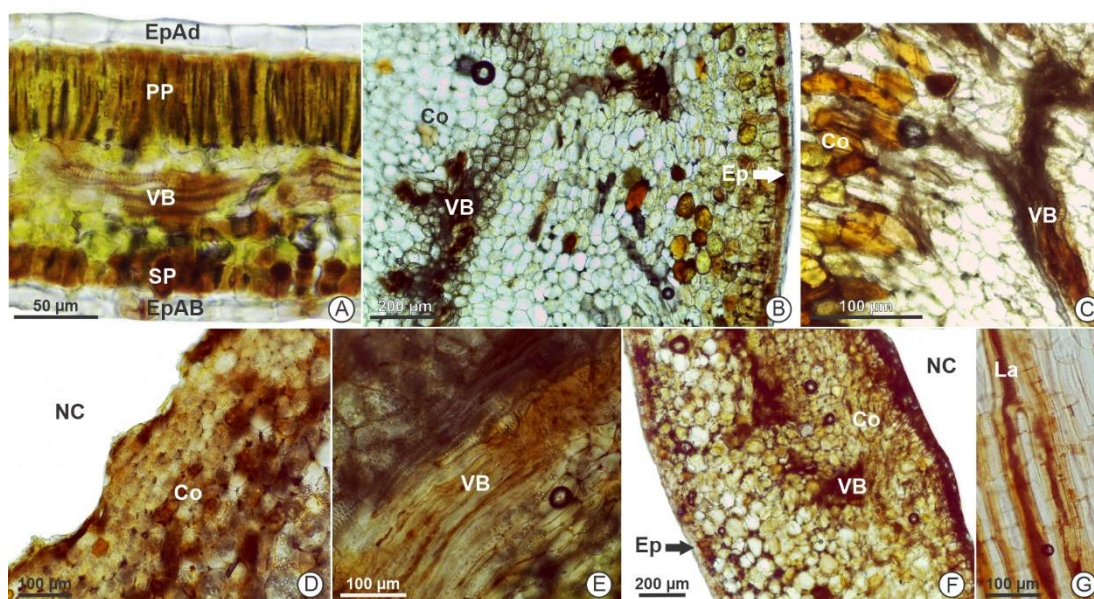
Figure 2. Histochemical detection of hydrogen peroxide with the DAB test in leaves (A) of *Sapium glandulosum* and in galls induced by *Neolithus fasciatus* in the young (B, C), mature (D, E) and senescent (F, G) stages. G- Laticifer. * Brown coloration shows positive reaction. Abbreviations: Ep – Epidermis; EpAd – Epidermis on the adaxial face; EpAb – Epidermis on the abaxial face; PP – Palisade parenchyma; SP – Spongy parenchyma; VB - Vascular bundle; Co – Cortex; NC – Nymphal camera; La – Laticifer.

3.2 ROS and lipid peroxidation

The concentration of hydrogen peroxide (H_2O_2) was higher in leaves (422.96 nmol g^{-1} FW $\pm$ 65.07) than galls and equal between the stages of development, i.e. young galls (207.63 nmol g^{-1} FW $\pm$ 34.67), mature galls (213.24 nmol g^{-1} FW $\pm$ 55.37) and senescent galls (232.27 nmol g^{-1} FW $\pm$ 42.41) (Figure 3A). The superoxide anion (O_2^-) was highest in the young galls (3.23 nmol g^{-1} FW $\pm$ 1.85), followed similarly by

mature galls (1.65 nmol g^{-1} FW $\pm$ 0.75) and senescent galls (1.30 nmol g^{-1} FW $\pm$ 0.51) and lower in leaves (0.78 nmol g^{-1} FW $\pm$ 0.15) (Figure 3B).

The lipid peroxidation, evaluated by malondialdehyde (MDA) concentration, was higher in leaves (0.10 nmol g^{-1} FW $\pm$ 1.42^{e-2}) and senescent galls (0.08 nmol g^{-1} FW $\pm$ 2.74^{e-3}), being lower in young galls (0.05 nmol g^{-1} FW $\pm$ 1.06^{e-2}) and mature galls (0.05 nmol g^{-1} FW $\pm$ 4.69^{e-3}) (Figure 3C).
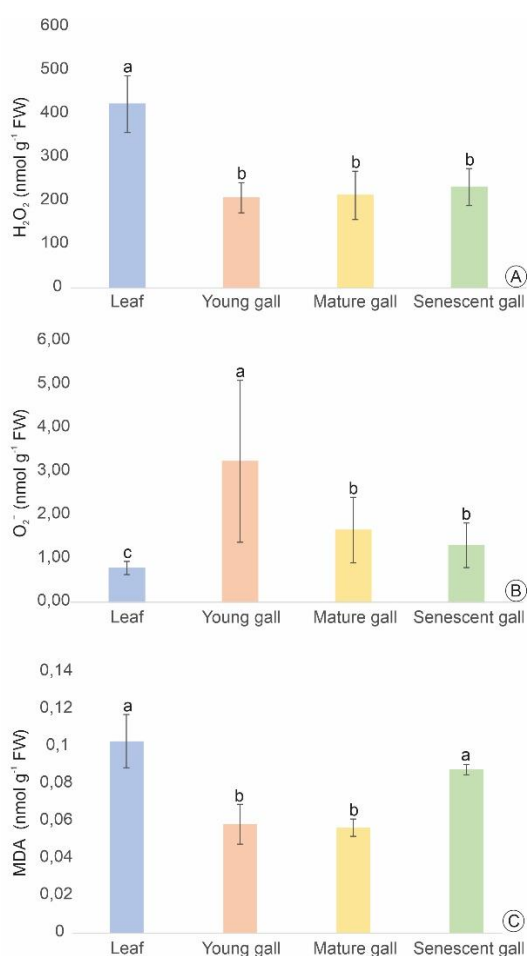
Figure 3. Quantification of oxidative stress in leaves of *Sapium glandulosum* and in the galls induced by *Neolithus fasciatus* in the young, mature, and senescent stages. A- Concentration of hydrogen peroxide (H_2O_2); B – Concentration of superoxide anion (O_2^-) and C – Concentration of malondialdehyde (MDA). Data were represented as mean $\pm$ standard deviation. *Means followed by equal letters do not indicate difference by Tukey's test, at 5% significance.

3.3 Antioxidant system analyses and phenolics

The enzymatic activity in leaves was different in relation to the galls (Figure 4). The catalase activity (CAT) was higher in the leaves (34.3^{e-6} µmol min^{-1} mg^{-1} prot $\pm$ 5.06^{e-6}) than in all stages of galls, reaching lower activity in the senescent galls (5.08^{e-7} µmol min^{-1} mg^{-1} prot $\pm$ 1.83^{e-6}) (Figure 4A). The activity of ascorbate peroxidase (APX) and peroxidase (POX) were similar, and the values found in the leaves were higher than in all stages of galls (Figure 4B, C). For superoxide dismutase (SOD), the young, mature and senescent galls had higher values than leaves (13.9^{e-4} µmol min^{-1} mg^{-1} prot $\pm$ $2.64e^{-4}$), as well as similar to each other (29.7^{e-4} µmol min^{-1} prot $\pm$ 3.68^{e-4}, 31.7^{e-4} µmol min^{-1} mg^{-1} prot $\pm$ 3.93^{e-4}, 28.3^{e-4} µmol min^{-1} mg^{-1} prot $\pm$ 3.30^{e-4}, respectively) (Figure 4D). The phenolic compound contents showed similar results between leaves and galls at all stages of development (mg/kg) (Figure 4E).
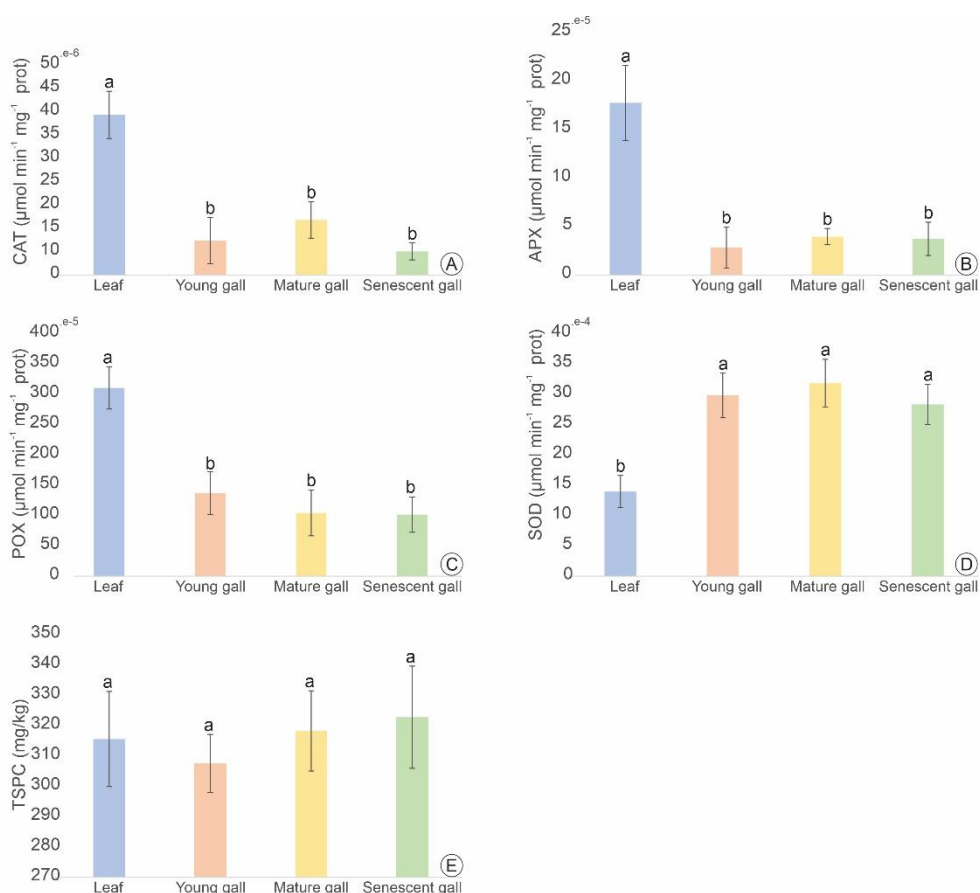
Figure 4. Enzymatic activity and phenolic contents in leaves of *Sapium glandulosum* and galls induced by *Neolithus fasciatus* in the young, mature, and senescent stages. A- Catalase activity (CAT); B- Ascorbate peroxidase activity (APX); C- Activity of peroxidase enzymes (POX); D- Superoxide dismutase activity (SOD); E – Total soluble phenolic compounds (TSPC). Data were represented as mean ± standard deviation. *Means followed by equal letters do not indicate difference by Tukey's test, at 5% significance.

3.4 Pigment content, chlorophyll a fluorescence and relative water content

The total chlorophyll values were higher in leaf than galls in the different stage of development (Table 1). The chlorophyll a/b ratio showed similar results between leaf, mature galls, and senescent galls, being higher than the young galls (Table 1). The total concentration of carotenoids was higher in the leaf, followed similarly between mature and senescent galls and lower in the young galls (Table 1). The chlorophyll/carotenoid ratio was higher for the leaf, with lower ratio for the mature and senescent galls (Table 1). The relative water content was higher in young galls than leaves, mature and senescent galls (Table 1).

Table 1. Chloroplastid pigments and relative water content in *Sapium glandulosum* leaves and galls induced by *Neolithus fasciatus* in the young, mature and senescent stages. Data were represented as mean ± standard deviation.

	Total chlorophyll ($\mu g\ g^{-1}$MF)	Chlorophyll (*a/b*)	Total carotenoids ($\mu g\ g^{-1}$MF)	Total chlorophyll/carotenoids	Relative water content (RWC) (%)
Leaf	$3311,0 \pm 502,6^a$	$4,1 \pm 0,3^a$	$273,7 \pm 35,9^a$	$12,0 \pm 0,6^a$	$63,32 \pm 5,33^b$
Young gall	$157,2 \pm 53,4^b$	$3,3 \pm 0,8^b$	$48,3 \pm 16,1^c$	$3,29 \pm 0,5^b$	$84,4 \pm 5,56^a$
Mature gall	$232,4 \pm 93,9^b$	$3,8 \pm 0,5^a$	$121,8 \pm 43,4^b$	$1,8 \pm 0,1^c$	$73,74 \pm 8,02^b$
Senescent gall	$190,2 \pm 54,8^b$	$3,5 \pm 0,5^a$	$95,6 \pm 20,0^b$	$1,9 \pm 0,1^c$	$63,24 \pm 12,82^b$

* Means followed by equal letters in the same column indicate no difference by Tukey's test, at 5% significance.

The minimum fluorescence of PSII in the state adapted to the dark (F_0) had higher values for the mature galls, followed equally by the leaf and young galls, and the senescent galls (Table 2). The maximum fluorescence of PSII in the dark-adapted state (F_M) was higher in the mature galls, followed similarly by the leaf and young galls, in addition to a lower value in the senescent galls (Table 2). The quantum yield of photosystem II in steady state adapted to light, the (F'm-F)/F'm, had similar values for all treatments (Table 2). The maximum quantum yield of photosystem II (Fv/Fm)

presented similar values for the treatments except for senescent galls (Table 2). The non-photochemical dissipation of energy (NPQ) was higher in the leaf, followed equally by the young and mature galls, and finally by the senescent galls (Table 2). The steady-state fluorescence decline ratio (R_{fd}), an empirical parameter used to assess plant vitality, was higher in leaf, followed by young galls, and being similarly lower in mature and senescent galls (Table 2).

Table 2. Chlorophyll *a* fluorescence in leaves of *Sapium glandulosum* and its galls induced *by Neolithus fasciatus* in the young, mature, and senescent stages. Data were represented as mean ± standard deviation.

| | Leaf | Gall | | |
	Mature	Young	Mature	Senescent
F_0	$59,04 \pm 4,78^b$	$65,30 \pm 9,48^{ab}$	$71,91 \pm 16,29^a$	$52,06 \pm 10,68^b$
F_M	$230,12 \pm 28,16^b$	$228,44 \pm 57,11^b$	$293,02 \pm 101,44^a$	$140,25 \pm 52,45^c$
Fv/Fm	$0,74 \pm 0,03^a$	$0,69 \pm 0,09^a$	$0,73 \pm 0.06^a$	$0,59 \pm 0,10^b$
$(F'_m\text{-}F)/F'_m$	$0,46 \pm 0,04^a$	$0,48 \pm 0,08^a$	$0,56 \pm 0,08^a$	$0,48 \pm 0,11^a$
NPQ	$2,32 \pm 0,31^a$	$1,56 \pm 0,25^b$	$1,22 \pm 0.29^b$	$0,63 \pm 0,26^c$
R_{fd}	$3,04 \pm 0,19^a$	$2,07 \pm 0,27^b$	$1,55 \pm 0,36^c$	$1,02 \pm 0,35^c$

Abbreviations: F_0- Minimal fluorescence of PSII in the state adapted to the dark; Fm- maximum fluorescence of PSII in the state adapted to the dark; Fv/Fm- maximum quantum yield of photosystem II; (F'm–F')/F'm- PSII operational efficiency; NPQ- Non-photochemical quenching during light adaptation; R_{fd}- Steady-state fluorescence decline ratio. * Means followed by equal letters on the same line do not indicate difference by Tukey's test, at 5% significance.

4. DISCUSSION

4.1 Oxidative stress and antioxidant activity

In the galls of *S. glandulosum*, the hydrogen peroxide occurred in few tissues, mostly in the feeding site of the gall-inducer, and in higher amount in leaves than galls. These data are contrary to what is expected, which can demonstrate that in gall there is intracellular homeostatic balance and effective control of the ROS levels by the activity of an antioxidant system (Foyer & Noctor 2009). The galls of *Aspidosperma australe* (Apocynaceae) and *A. tomentosum* (Apocynaceae) had similar results, which were

associated with the lower stress caused by the sucking apparatus of the gall insects (Kuster *et al*. 2022), which is like that of *Neolithus fasciatus*. The senescence of the galls of *S. glandulosum* seems to be related to increased lipid peroxidation, which triggers a cumulative deterioration of membranes, as reported for senescent leaves of *Nicotiana tabacum* L. (Solanaceae) (Dhindsa *et al*. 1981). Senescence and lipid peroxidation have been associated in different plant species, such as *Brassica oleracea* L. (Brassicaceae) (Zhuang *et al*. 1995) and *B. oleracea* L. (Brassicaceae) (Cheour *et al*. 1992).

Superoxide (O_2^-) is the first ROS generated after the reduction of molecular O_2 and is considered to have strong reactivity and oxidizing capacity (Saibi & Brini 2018). That ROS was in higher amount in young galls of *S. glandulosum* than to the other stages, since after induction the galls increase growth rates and cellular (Isaias *et al*. 2015). O_2^- arises in several cellular compartments, but mitochondrial respiration is one of the main producers of this ROS (Saibi & Brini 2018), which justifies its high concentration in the young galls of *S. glandulosum*. The high concentration of superoxide was not materialized in a secondary stress in galls, labeled here by malondialdehyde, which may be related to dissipation via SOD, which generally prevents lipid peroxidation (Gill & Tuteja 2010).

The accumulation of phenolics has been reported for galls induced by insects and in different host plant species, such as in the galls induced by *Leptocybe invasa* (Hemynoptera) in *Eucalyptus camaldulensis* (Myrtaceae) (Isaias *et al*. 2018), reducing the harmful effects of free radicals (Gottlieb & Kaplan 1993). For this reason, the production of phenolic compounds in galls are typically hyper-stimulated by the gall-inducers, as demonstrated for galls of *Miconia albicans* and *M. ibaguensis* (Melastomataceae) induced by *Ditylenchus gallaeformans* (Nematoda) (Ferreira *et al*.

2018). However, the storage of phenolic compounds was similar between the leaves and all stages of development of the galls of *S. glandulosum*, which may reflect the low stress pointed out for the galls. Despite the similarity, Rosa (2022) demonstrated that phenolics occurred mainly in the vascular bundles of mature galls of *S. glandulosum*, indicating that phenolic production sites are associated with tissues with higher hydrogen peroxide labeling.

Antioxidant enzymes such as catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and superoxide dismutase (SOD) are important in protecting membranes because they prevent lipid peroxidation and act in defense against reactive oxygen species (ROS) (Purvis & Shewfelt 1993). These enzymes remove peroxidized fatty acids from the membrane (Shewfelt & Brenda 2000) and thus prevent elevated oxidative stress (Barbosa *et al*. 2014). Under normal conditions, ROS molecules can be scavenging by the antioxidant defense mechanisms, but the oxidative balance can be affected by stress triggered by abiotic and biotic factors, which elevate oxidative stress through the production of H_2O_2 (Gill & Tuteja 2010).

In the current work, a higher activity of antioxidant enzymes in the *S. glandulosum* leaves was reported compared to the three stages of gall development, possibly acting on the dissipation of the strongly labeled H_2O_2 in its chlorophyllous tissue. The photosynthetic process is responsible for the production of ROS in chloroplasts, mainly by the reduction of the O_2 product from the water photolyzes, which can produce O_2^- and H_2O_2 (Khorobrykh *et al*. 2020). Chloroplasts have ROS elimination systems to prevent damage to the photosynthetic process (Asada 2008; Triantaphylidès *et al*. 2008), represented by the enzymes CAT, APX and POX in the *S. glandulosum* leaves and which apparently do not avoid the high levels of lipid peroxidation reported here. The increase in these enzymes is related to the dissipation of

biotic and abiotic stress in plants, as demonstrated in the leaves of *Urochloa ruziziensis* (Poaceae) under severe water stress (Bulegon *et al*. 2016). The lower activity of these enzymes in the galls may be related to the weak histochemical labeling of H_2O_2 in their tissues, possibly linked to the low oxidative stress promoted by the inducer's feeding habit (see Kuster *et al*. 2022).

The SOD acts at the beginning of antioxidant defense, mainly dismuting the superoxide anion O_2^- and decreasing the formation of the hydroxyl radical (OH^-), which is one of the most offensive ROS in lipid degradation (Gill & Tuteja 2010). SOD was found in higher activity in galls in its three stages of development in relation to leaves, proving to be the main system of ROS dissipation in the *S. glandulosum* galls. This increased SOD activity may cause the other antioxidant enzymes to show a reduction in demand, as it is acting as the first line of defense (Saibi & Brini 2018). Associated with the action of SOD, there was a high labeling of H_2O_2 in the cell walls of the different tissues of young galls, which may come from the dismutation of O_2^{--}. H_2O_2 is a promoter of plant development, mainly by stimulating cell expansion (Isaias *et al*. 2015; Schmidt *et al*. 2016) and can therefore be a signal of the development of *S. glandulosum* galls. That cell expansion may also be driven by higher RWC in young galls, which generates a higher turgor pressure (Taiz *et al*. 2017). In galls of *Cuscuta campestris* (Convolvulaceae) induced by the gall insect *Smicronyx sp*. nov. (Coleoptera: Curculionidae) there was also an increase in SOD in response to increased metabolism and maintenance of photosynthetic activity (Zagorchev *et al*. 2018). SOD activity is critical for the scavenging of superoxide radicals generated during photosynthesis, even under ideal conditions of redox homeostasis (Foyer & Shigeoka 2011).

4.2 Chloroplastid pigments and chlorophyll a fluorescence

The induction of galls in leaves usually causes a reduction in the concentration of chloroplastid pigments, represented by chlorophyll and carotenoids (Rezende *et al.* 2018; Oliveira *et al.* 2017; Kuster *et al.* 2022). The pigment reduction of *S. glandulosum* galls follows the pattern reported for leaf galls of *Eugenia uniflora* induced by *Eugeniamya dispar* (Rezende *et al.* 2018) and for four leaf morphotypes induced in *Aspidosperma* spp. (Kuster *et al.* 2022). The drop in pigments is related to cellular hypertrophy and tissue hyperplasia (Mani 1964) and is usually associated with the dilution process, as reported for galls of *M. guianensis* (Oliveira *et al.* 2017). However, here the RWC was only higher in the young galls, being similar between the leaves and the other gall stages, which leads us to believe that the reduction in the chloroplastid pigments in the *S. glandulosum* galls is not due to a dilution process. Gall formation is based on the redifferentiation of cells and tissues through different cytohistological processes (Isaias *et al.* 2011; Oliveira *et al.* 2014), with the emergence of new morphofunctional compartments distinct from the pre-existing ones (Bragança *et al.* 2022). Thus, we believe that the change in tissue functionality is associated with the degradation of pigments in *S. glandulosum* and investment in new organelles and tissue structure. The reduction in chlorophyll concentration in the *S. glandulosum* galls was accompanied by increased initial fluorescence (F_0) and maximum fluorescence (Fm) in mature galls, as well as demonstrated for galls of *E. uniflora* (Rezende *et al.* 2018). Higher values of F_0 and Fm may indicate the biotic stress generated by gall induction, which may be associated with damage to photosystem II (PSII) and/or pairing in the excitation energy transfer of the antenna complex (Bolhar-Nordenkampf *et al.* 1989).

The lower concentration of carotenoids in the young galls of *S. glandulosum* compared to the other stages of development demonstrates loss in the process of energy

dissipation (Demming-Adams & Adams 1996; Taiz *et al*. 2017), which may be associated with prominent cytohistological reorganization at this stage. Lower carotenoid concentrations in young versus mature galls have also been reported for leaf galls induced by *Pseudophacopteron longicaudatum* on *Aspidosperma tomentosum* (Martini *et al*. 2020). That tissue restructuring also reduced non-photochemical quenching (NPQ) and tissue vitality, demonstrated by Rfd, in the gall relative to the leaf, as demonstrated for galls of *M. guianensis* (Oliveira *et al*. 2017). These parameters were more affected by gall senescence, where degradation of nuclei and encapsulation of organelles for cellular digestion have already been reported (Oliveira *et al*. 2010).

Reduction of maximum PSII quantum yield (F_v/F_m) and PSII operational efficiency ($(F'_m–F')/F'_m$) appears to be a usual response to leaf gall induction, as demonstrated for leaf galls of *Eugenia uniflora* (Rezende *et al*. 2018), *Matayba guianensis* (Oliveira *et al*. 2017), and *Aspidosperma tomentosum* (Martini *et al*. 2020). The reduction in quantum efficiency in galls is usually associated with decreased chlorophyll content, as demonstrated here, which is the site of light absorption and chemical energy formation (Oliveira *et al*. 2017). As well, the decrease in the mechanism of energy capacitation and dissipation may also be related to the reduction of quantum yield in PSII, as demonstrated for galls induced by *D. gallaeformans* (Nematoda) in leaves of *Miconia albicans* (Melastomataceae) (Ferreira *et al*. 2018). However, the galls of *S. glandulosum* are out of the ordinary, maintaining the quantum yield of the photosystem, being similar to that found in galls of Cecidomyiidae in leaves of *Clusia arrudae* (Clusaceae), which maintained the quantum yield similar to the non-galled organ (Fernandes *et al*. 2010). The maintenance of quantum yield in galls may be a compensatory response, decreasing this negative effect of galls on plant development (Fay *et al*. 1993).

5. CONCLUSION

The development of *S. glandulosum* galls was mediated by a low oxidative stress, controlled mainly by SOD activity as the first line of antioxidant defense. Unexpectedly, the leaf presented a high oxidative stress associated with photosynthetic tissue, even with high investment in antioxidant enzymes. This stress seems to have negatively impacted the quantum yield of photosynthesis, with performance similar to that of non-senescent galls, even with the reduction of chlorophyll concentration in galls.

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