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EFFECTS OF GLYPHOSATE ON GERMINATION AND PHOTOSYNTHESIS IN *Prosopis alba* G.: A BIOCHEMICAL APPROACH

EFEITOS DO GLIFOSATO NA GERMINAÇÃO E NA FOTOSSÍNTESE EM Prosopis alba G.: UMA ABORDAGEM BIOQUÍMICA

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ABSTRACT

In recent decades, the phytogeographic region of the Western Chaco has been subjected to heavy deforestation. The native forest was gradually replaced by agricultural crops using high doses of herbicides. Glyphosate is the most widely used herbicide, and its impact on the surrounding native flora is unknown. The aim of this work was to determine the effect of glyphosate on the germination of *Prosopis alba* seeds and the photosynthesis of seedlings. Seeds were placed between paper towels, moistened with solutions of 0, 10, 20, 20, 30 and 40 mg a.i. glyphosate l^{-1}, in a growth chamber at 25 ºC and a 12 h photoperiod. The percentage of germinated seeds and the mean germination time were calculated. The respiratory rate was measured in these seeds, and the activity of complexes I and III of the respiratory chain was quantified. The shikimate concentration and antioxidant response of the seeds were also quantified. Chlorophyll fluorescence emission variables were measured in the cotyledons. It was concluded that glyphosate inhibits germination in *P. alba* seeds and decreases the speed of the process. This effect can partly be explained by inhibition of respiration, mainly at the level of complex III of the mitochondrial electron transport chain. It is also due to oxidative stress produced by the herbicide since the antioxidant response of the seeds fails to compensate for the high production of reactive oxygen species. Glyphosate inhibits the photochemical stage of photosynthesis on *P. alba* cotyledons.

KEYWORDS: **Germination, Herbicides, Oxidative stress, Photosynthesis, Respiration.**

RESUMO

Nas últimas décadas, a região fitogeográfica do Chaco Ocidental tem sofrido forte desmatamento. A floresta nativa foi substituída por lavouras agrícolas, com uso de altas doses de herbicidas. O glifosato é o herbicida mais utilizado, e o seu impacto na flora nativa circundante é desconhecido. O objetivo deste trabalho foi determinar o efeito do glifosato na germinação de sementes de *Prosopis alba*, e na fotossíntese das plântulas. As sementes foram colocadas entre toalhas de papel umedecidas com soluções de 0, 10, 20, 30 e 40 mg i.a. glifosato l^{-1}, em câmara de crescimento a 25º C e fotoperíodo de 12 horas. Foram calculados a porcentagem de sementes germinadas e o tempo médio de germinação. Nessas sementes foi medida a taxa respiratória e quantificadas as atividades dos complexos I e III da cadeia respiratória. Também foram quantificadas a concentração de chiquimato e a resposta antioxidante das sementes. Nos cotilédones foram medidas variáveis de emissão de fluorescência da clorofila a. Conclui-se que o glifosato inibe a germinação de sementes de *P. alba* e retarda este processo. Tal efeito pode ser explicado, em parte, pela inibição no nível do complexo III da cadeia de transporte de elétrons mitocondrial. Também se deve ao estresse oxidativo produzido pelo herbicida, uma vez que, a resposta antioxidante das sementes não pode compensar a alta produção de espécies reativas de oxigênio. O glifosato inibe a etapa fotoquímica da fotossíntese nos cotilédones de *P. alba*.

PALAVRAS-CHAVE: **Germinação, Herbicidas, Estresse oxidativo, Fotossíntese, Respiração.**

INTRODUCTION

In recent decades, the phytogeographic region of the Western Chaco has been subjected to heavy deforestation. Thus, a change in land use has been observed, and the native forest has been gradually replaced by agricultural crops (HOYOS et al., 2013). The sustained increase in the international price of commodities has accelerated this process and encouraged the cultivation of transgenic soybean (CÁCERES et al., 2015). The management of this crop includes the use of high doses of herbicides, mainly glyphosate (PIQUER-RODRÍGUEZ et al., 2015).

Herbicides can be transported by wind towards the surrounding native forest, thus reaching non-target species. This phenomenon, known as drift, can lead to the death of sensitive species and jeopardize the biodiversity of these ecosystems (DUPONT et al., 2018).

Recently, FERREIRA et al. (2017) studied the effect of glyphosate on 23 herbaceous and shrub species native to the Western Chaco. Their results showed that a dose of 25% of the recommended field rate was lethal or sublethal for all of them (50% of the species studied manifested severe toxicity and 75% showed reduced growth).

Algarrobo blanco (*Prosopis alba*) is a native tree species of the Western Chaco; its wood is highly valued for furniture production, and its fruits are consumed by wildlife and livestock (MELONI et al., 2019). The degree of tolerance of *P. alba* to glyphosate and the impact of this herbicide on germination and seedling development of this species are unknown.

Glyphosate acts by inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), which is involved in the shikimic acid pathway. This metabolic process leads to the synthesis of aromatic amino acids (ALCÁNTARA DE LA CRUZ et al., 2016).

Germination is a critical stage for the establishment of seedlings in forest ecosystems. The effect of glyphosate on germination has been insufficiently studied, and the results obtained have been contradictory. GOMES et al. (2017a) demonstrated that glyphosate inhibits the germination of *Dimorphandra wilsonii*, a species native to the Brazilian Cerrado. On the other hand, glyphosate does not affect the percentage of maize germination, but decreases the speed of the process (GOMES et al., 2019a).

Glyphosate can affect the physiology of seedlings of forest species, inhibiting photosynthesis and altering ionic homeostasis and nitrogen metabolisms, among others (CARVALHO et al., 2018; GOMES et al., 2014). Glyphosate can also lead to the accumulation of reactive oxygen species (ROS). ROS are involved in the mobilization of seed reserves as well as in the signaling in response to environmental cues. At high concentrations, they can cause severe damage to metabolism (VERMA et al., 2015).

The aim of this work was to determine the effect of glyphosate on the germination of *P. alba* seeds and the photosynthesis of seedlings.

MATERIAL AND METHODS

Seed collection area

Prosopis alba fruits were harvested from randomly selected trees at the experimental station of the Instituto Nacional de Tecnología Agropecuaria, Santiago del Estero, Argentina (28º 3' S, 64º 14' E). The experimental station has an area of approximately 6 km^2 with different types of vegetation: native forest, grasslands and shrublands. It is located in the phytogeographic region of the Western Chaco and has a subtropical climate with a dry season. The average annual temperature is 26^oC, and the annual rainfall is 574 mm (IBÁÑEZ et al., 2021).

Seed germination trials

Seeds were manually extracted and selected by size and uniform color. They were then disinfected with 2.5% sodium hypochlorite for 10 min, followed by three washes in sterile distilled water. To facilitate the imbibition of the seeds, a cut was made with pliers at the opposite end of each embryo. Four replications of 50 seeds were placed to germinate between paper towels moistened with 10 ml of distilled water (control) or glyphosate solutions of 10, 20, 30 and 40 mg a.i. l^{-1}. The germination towels were rolled, covered with polyethylene bags to minimize water loss by evaporation and placed vertically in a growth chamber at 25 ^oC with a 12 h photoperiod. Seeds in which the cotyledons emerged were considered germinated. Germination percentage and mean germination time (MGT) were calculated according to NICHOLS & HEIDECKER (1996).

Respiration measurements and determinations of the activity of complexes I and III of the mitochondrial electron transport chain

Respiration measurements were made 48 h after the treatments were initiated. A total of 1.5 g of seeds was placed in 5 ml vials containing 8 ml of 8 mM HEPES buffer,

pH 7.2. The O_2 consumption was measured using a TBR1025 sensor (World Precision Instruments, Sarasota, Florida, USA). This sensor consists of silver and platinum electrodes. The measurements were performed following the methodology described by Pandey et al. (2019).

To determine the activity of complexes I and III of the mitochondrial electron transport chain, seeds were homogenized in 200 mM potassium phosphate buffer, pH 7.5. The activities of complexes I (NADH: ubiquinone oxidoreductase) and III (ubiquinol-cytochrome c reductase) were determined spectrophotometrically according to the techniques described by Estornell et al. (1993) and Birch-Machin et al. (1993), respectively.

Shikimate determinations and oxidative stress variables

Seeds were arranged under the same conditions and treatments described in the germination assays.

Shikimate was quantified using the technique reported by Singh & Shaner (1998), with some modifications. Seeds were homogenized with a pestle and mortar in 0.25 N HCl. The homogenate was then centrifuged at 25,000 x g and 5 °C for 20 min. Aliquots of 40 µl of the supernatant were collected, to which 500 µl of 1% periodic acid solution was added. After incubation for 2 h, 500 µl of 1 N NaOH solution and 300 µl of 0.1 M glycine were added. Vigorous mixing was done, and absorbance was read at 380 nm. Concentrations were expressed in µmol g^{-1} FW.

The $O_2^{\cdot-}$ concentration was quantified by nitrite formation from hydroxylamine in the presence of superoxide (ELSTNER & HEUPEL, 1976). Results were expressed in nmol min^{-1} g^{-1} FW.

The H_2O_2 concentration was determined by the technique described by ZHOU et al. (1997) using N-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red). The enzymatic oxidation of Amplex Red was quantified spectrophotometrically, reading absorbance at 560 nm. Results were expressed as µmol H_2O_2 g^{-1} FW.

Malondialdehyde was extracted with trichloroacetic acid and quantified spectrophotometrically according to the method reported by Heath & Paker (1968). The concentration was calculated using an absorption coefficient of 155 mM^{-1} cm^{-1}. Results were expressed as µmol g^{-1} FW.

To determine the enzymatic activities, seeds were homogenized in phosphate buffer, pH 7.0. The homogenate was centrifuged at 15,000g and 5 °C for 20 minutes. The supernatant was used to determine protein concentration, according to the technique developed by Bradford (1976), and enzyme activities.

Ascorbate peroxidase activity (APX, EC 1.11.1.11) was determined by measuring ascorbate oxidation at 290 nm, using the technique provided by Nakano & Asada (1981). APX activity was expressed as µmol ascorbate mg^{-1} protein min^{-1}.

Catalase activity (CAT; EC 1.11.1.6) was determined through the oxidation of H_2O_2 at 240 nm, using a molar extinction coefficient of 36 M^{-1} cm^{-1}, according to the technique described by HAVIR & MCHALE (1987). The CAT activity was expressed as µmol H_2O_2 mg^{-1} $protein^{-1}$ min^{-1}.

The superoxide dismutase activity (SOD, EC 1.15.1.1) was determined according to the method used by Giannopolitis & Ries (1977). For this purpose, the photoreduction inhibition of nitro blue tetrazolium chloride was quantified at 540 nm. One unit of SOD consisted of the amount of enzyme required to inhibit by half the photoreduction of nitro blue tetrazolium chloride. SOD activity was expressed as U mg^{-1} protein min^{-1}.

Chlorophyll a fluorescence emission measurement

Chlorophyll fluorescence emission measurements were made on cotyledons of germinated seeds. Measurements were performed with a portable fluorometer (LI-6400-40; Li-Cor Inc.). The light-saturation pulse method was used in light-adapted cotyledons and 1 h of darkness (SCHREIBER et al., 1994). Pulse intensity and duration were 8,000 µmol m^{-2} s^{-1} and 0.8 s, respectively. The following variables were calculated: maximum PSII quantum efficiency (F_v/F_m), photochemical quenching (qP) and non-photochemical quenching (NPQ), according to the equations proposed by Genty et al. (1989), Kramer et al. (2004) and Bilger & Björkman (1995), respectively.

Experimental design and statistical analysis

A completely randomized experimental design with four replicates was used. The experimental unit was represented by a germination towel with 50 seeds. Results were analyzed with ANOVA and Tukey's test.

RESULTS AND DISCUSSION

The germination of *P. alba* was inhibited from a dose of 30 mg a.i. glyphosate l^{-1}. The maximum inhibition was recorded at the dose of 40 mg a.i. glyphosate l^{-1}, with a reduction of 30% with respect to the control (Figure 1A). The speed of the process, measured through the MGT, was more sensitive than the final percentage of germinated seeds (Figure 1 B). As the dose was increased,

germination was slower, with a concomitant increase in the MGT. Thus, while in the control the MGT was approximately 4 days, at the highest dose it approached to 9 days.

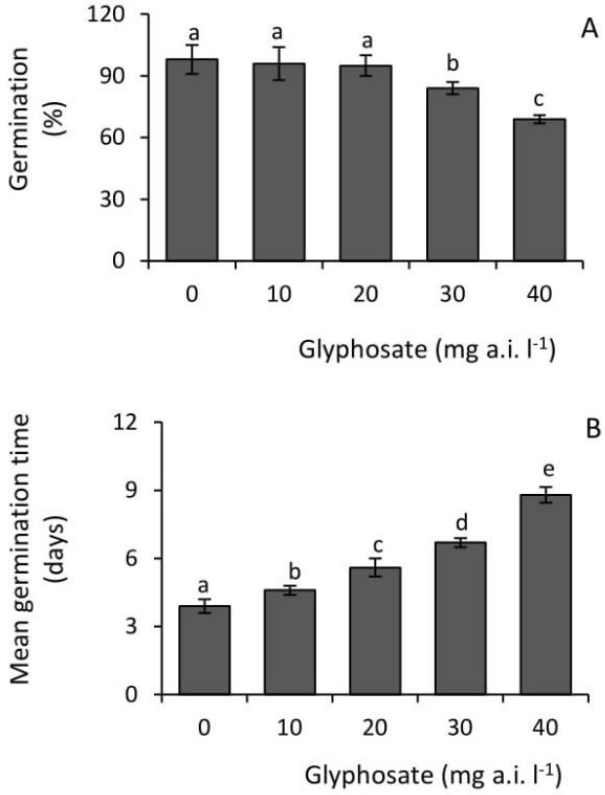

Figure 1. Germination (A) and mean germination time (B) in *P. alba* seeds treated with increasing doses of glyphosate. Different letters indicate significant differences by Tukey's test at 5%.

Coinciding with the behavior observed in the MGT, all glyphosate doses increased the concentration of shikimate in *P. alba* seeds (Figure 2). This result indicates that doses of 10 to 40 mg a.i. glyphosate l^{-1} inhibited the EPSPS enzyme. On the other hand, the increase in shikimate concentration is a typical response of glyphosate-sensitive species (SCHRÜBBERS et al., 2014).

To our knowledge, this is the first report on the effect of glyphosate on *P. alba* germination. For this reason, we investigated its effect on the respiratory metabolism. Respiration is a very important metabolic process during germination because it provides energy and carbon skeletons for biosynthetic pathways (GOMES et al., 2014).

As shikimate concentration in seeds increased, the respiration rate decreased (Figure 3A). Respiration in seeds treated with 10 and 40 mg a.i. glyphosate l^{-1} decreased by 14% and 41% compared to the control, respectively.

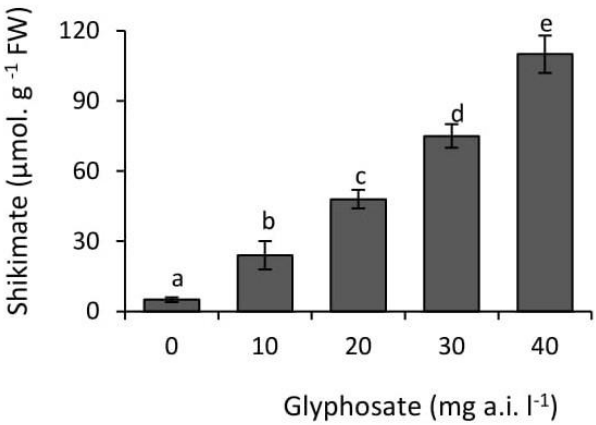

Figure 2. Concentration of shikimate in *P. alba* seeds treated with increasing doses of glyphosate. Different letters indicate significant differences by Tukey's test at 5%.

Respiration consists of four stages: glycolysis, Krebs cycle, mitochondrial electron transport chain and oxidative phosphorylation. We studied the effect of glyphosate on the activity of protein complexes I and III in the mitochondrial electron transport chain. The activity of both complexes was inhibited by the herbicide, but the effect was more pronounced on complex III (Figures 3B and 3C). The activity of complex I was inhibited from 20 mg a.i. glyphosate l^{-1}; at the dose of 40 mg a.i. glyphosate l^{-1}, its activity was 33% lower than in the control. In contrast to this response, the activity of complex III was inhibited from 10 mg a.i. glyphosate l^{-1}; at the dose of 40 mg a.i. glyphosate l^{-1}, its activity was 62% lower than in the control.

Gomes et al. (2017a) reported similar results on seeds of *Dimorphandra wilsonii*, a leguminous tree species native to the Brazilian Cerrado. In that species, doses of 5 to 50 mg a.i. glyphosate l^{-1} produced a drastic inhibition in germination. These authors also observed that glyphosate inhibited seed respiration, especially at the level of complex III activity. Similarly, doses of 24 to 100 mg a.i. glyphosate l^{-1} produced a 30% reduction in sorghum germination. This effect was due to inhibition in the *de novo* synthesis of gibberellins and in the respiratory rate (GOMES et al., 2019b).

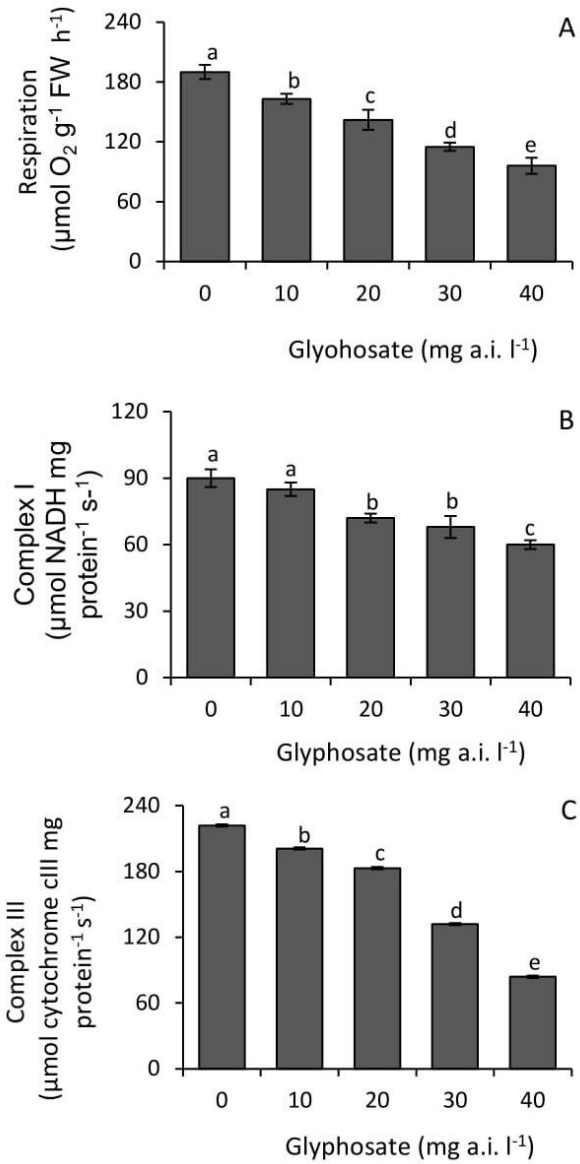

Figure 3. Respiratory rate (A) and activities of complexes I (B) and III (C) of the mitochondrial electron transport chain in *P. alba* seeds treated with increasing doses of glyphosate. Different letters indicate significant differences by Tukey's test at 5%.

In response to glyphosate, *P. alba* seeds increased the activity of enzymes involved in the ROS detoxification (Table 1). The APX activity increased from the dose of 30 mg a.i. glyphosate l^{-1}, whereas the activities of CAT and SOD enzymes increased from 20 mg a.i. glyphosate l^{-1}.

Table 1. Activities of ascorbate peroxidase (APX, µmol ascorbate mg^{-1} protein min^{-1}), catalase (CAT, µmol H_2O_2 mg^{-1} $protein^{-1}$ min^{-1}) and superoxide dismutase (SOD, U mg^{-1} protein min^{-1}) enzymes in *P.alba* seeds treated with increasing doses of glyphosate. Values represent means ± SD of four replicates.

Glyphosate (mg a.i. l^{-1})	APX	CAT	SOD
0	0,18±0,02 a	31,2±1,30 a	1,21±0,09 a
10	0,20±0,01 a	30,4±0,92 a	1,33±0,07 a
20	0,17±0,03 a	36,7±2,57 b	2,84±0,14 b
30	0,25±0,01 b	41,0±1,45 c	3,98±0,11 c
40	0,41±0,04 c	60,9±2,61 d	5,17±0,23 d

Means followed by the same letter in the column do not differ statistically at p<0.05 (Tukey's test).

Despite the increase in APX, CAT and SOD enzyme activities, glyphosate produced oxidative stress in *P. alba* seeds, increasing superoxide radical ($O_2^{\cdot-}$) and H_2O_2 concentrations (Table 2). Coinciding with the increase in ROS concentrations, there was a strong increase in the concentration of MDA (Table 2), the product of lipid peroxidation.

Table 2. Concentrations of superoxide radical ($O_2^{\cdot-}$, nmol min^{-1} g^{-1} FW), hydrogen peroxide (H_2O_2, µmol g^{-1} FW) and malondialdehyde (MDA, µmol g^{-1} FW) in *P. alba* seeds treated with increasing doses of glyphosate. Values represent the means ± SD of four replicates.

Glyphosate (mg a.i. l^{-1})	$O_2^{\cdot-}$	H_2O_2	MDA
0	9,2±0,3 a	10,1±0,2 a	21,1±3,2 a
10	17,5±0,5 b	13,6±0,1 b	32,9±2,5 b
20	24,1±0,1 c	15,9±0,9 c	45,4±4,9 c
30	43,8±0,6 d	23,5±0,7 d	73,8±1.6 d
40	59,3±0,3 e	33,4±0,4 e	102,2±4,1 e

Means followed by the same letter in the column do not differ statistically at p<0.05 (Tukey's test).

High ROS production can damage macromolecules such as proteins, lipids and DNA. At the cellular level, lipid peroxidation can alter the selective permeability of membranes, with loss of solutes (VIEIRA et al., 2019). However, ROS also increase protein carbonylation, which increases their proteolysis, leading to the mobilization of these reserves during germination (JOB et al., 2005). Therefore, at low concentrations, ROS are essential to promote the signaling processes necessary for germination. At high concentrations, ROS are detrimental

since they can compromise membrane structure and biological functions.

Gomes et al. (2019a) reported that glyphosate decreased the germination rate in maize. This trend was accompanied by an increase in the activity of both ascorbate peroxidase and catalase antioxidant enzymes. According to these authors, such an increase in the antioxidant activity allowed maintaining constant ROS contractions, interfering with germination.

Gomes et al. (2017b) reported different germination responses of three soybean cultivars treated with glyphosate. The germination of cultivars BRS 284 and L 8307 RR was inhibited by glyphosate due to a significant increase in H_2O_2 concentrations. The germination of cultivar AS 3810 IORO was resistant to the herbicide, coinciding with low H_2O_2 production. According to these authors, ROS production occurs at the level of complex III of the mitochondrial inner membrane. Thus, the greater the inhibition of the activity of this protein complex, the greater the production of ROS.

Glyphosate inhibited the photochemical stage of photosynthesis in *P. alba* cotyledons. The F_v/F_m ratio decreased from the dose of 20 mg a.i. glyphosate l^{-1} (Table 3). This ratio represents the maximum PSII quantum efficiency, that is, the maximum efficiency with which light is absorbed by PSII and used for quinone A reduction. Under optimal growth conditions, it has a value close to 0.75; lower values indicate photoinhibition (LIMA-MELO et al., 2019). Meloni & Martinez (2021) also reported photoinhibition in *Eucalyptus camaldulensis* seedlings under simulated glyphosate drift. All glyphosate doses tested produced decreases in qP and NPQ values (Table 3).

Table 3. Maximum PSII quantum efficiency (F_v/F_m), photochemical quenching (qP) and non-photochemical quenching (NPQ) in *P. alba* cotyledons from seeds germinated in the presence of increasing doses of glyphosate. Values represent the means ± SD of four replicates.

Glyphosate (mg a.i. l^{-1})	F_v/F_m	qP	NPQ
0	0,74±0,09 a	0,81±0,03 a	3,22±0,17 a
10	0,76±0,11 a	0,73±0,09 b	2,68±0,21 b
20	0,65±0,06 b	0,56±0,05 c	2,13±0,19 c
30	0,52±0,03 c	0,48±0,02 d	1,09±0,13 d
40	0,26±0,01 d	0,27±0,01 e	0,62±0,18 e

Means followed by the same letter in the column do not differ statistically at $p<0.05$ (Tukey's test).

The qP values represent the proportion of open reaction centers; therefore, a decrease in them indicates lower photochemical efficiency (LIMA NETO et al., 2019). A decrease in NPQ values was also recorded. The increase in NPQ values constitutes a photoprotection mechanism, dissipating as heat the excess energy in the photochemical stage of photosynthesis. This dissipation occurs through the xanthophyll cycle and tends to prevent photoinhibition. The decrease in NPQ values shows a deficient dissipation of excess energy as heat, which could be given by a lower concentration of carotenoids (VIEIRA et al., 2019).

The analysis of the behavior of fluorescence emission variables allows inferring that 10 mg a.i. glyphosate l^{-1} produced a dynamic photoinhibition. This is because a decrease in qP was recorded, while the F_v/F_m ratio remained constant. From 20 mg a.i. glyphosate l^{-1}, the herbicide produced chronic photoinhibition, which is usually associated with damage to the D_1 protein of photosystem II (LIMA-MELO et al., 2019).

Freitas-Silva et al. (2020) studied the effect of glyphosate on the physiology of two neotropical species native to Brazil, *Handroanthus chysotricus* (very sensitive to herbicide) and *Garcinia gardneriana* (moderately sensitive to herbicide). Doses from 360 to 1440 g a.i. ha^{-1} inhibited net photosynthesis of *H. chysotricus*, with significant decreases in the relation F_v/F_m, qP and NPQ. In *G. gardneriana*, glyphosate did not affect net photosynthesis or chlorophyll a fluorescence variable. In *Pouteria torta*, doses greater than or equal to 25 g a.i. ha^{-1} of glyphosate reduced net photosynthesis and produced photoinhibition, which was manifested by a decrease in the F_v/F_m ratio. They also observed increased NPQ and a decrease in chlorophyll a and b concentrations (BATISTA et al., 2018).

CONCLUSIONS

It is concluded that glyphosate inhibits germination in *P. alba* seeds and decreases the speed of the process. This effect can be partly explained through the inhibition of respiration, mainly at the level of complex III of the mitochondrial electron transport chain. It is also due to oxidative stress produced by the herbicide since the antioxidant response of the seeds fails to compensate for the high production of ROS. Glyphosate inhibits the photochemical stage of photosynthesis in *P. alba* cotyledons.

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