UNIVERSIDADE FEDERAL DO PARANÁ
BIOFIX Scientific Journal v. 6 n. 1 p. 46-53 2021
DOI: dx.doi.org/10.5380/biofix.v6i1.77236

PHYSIOLOGICAL RESPONSES OF *Eucalyptus camaldulensis* (Dehnh.) TO SIMULATED GLYPHOSATE DRIFT

RESPOSTAS FISIOLÓGICAS DE Eucalyptus camaldulensis (DEHNH.) *À DERIVA SIMULADA DE GLIFOSATO*

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ABSTRACT

Weed control with glyphosate produces damages in plantations of *Eucalyptus camaldulensis*, although the involved physiological mechanisms have not been completely elucidated. This work aimed at assessing the physiological responses of *E. camaldulensis* to simulated glyphosate drift. Greenhouse trials were performed with four-month-old *E. camaldulensis* clone117 seedlings. The herbicide drift was simulated applying doses of 0; 43,2; 86,4; 172,8 and 345,6 g a.e. ha^{-1} glyphosate. Twenty-three days after the application, we measured gas exchange and chlorophyll a fluorescence. We also quantified Rubisco activity and indicator variables of oxidative stress. Glyphosate decreased carbon photosynthetic assimilation, increased non-photochemical quenching, induced stomatal closure, and increased photoinhibition. It also decreased Rubisco activity and increased photorespiration. The herbicide produced oxidative stress, and increased the activities in the enzymes catalase, ascorbate peroxidase, and superoxide dismutase, involved in the detoxification of reactive oxygen species. We concluded that glyphosate´s deleterious effects on the assimilation of CO_2 in *E. camaldulensis* are due to stomatal and non-stomatal effects. The decrease in Rubisco activity, the increase in photorespiration, and photoinhibition stand out among non-stomatal effects. The increase in the activity of the antioxidant system is insufficient to compensate for the production of H_2O_2 in photorespiration, which damages the photosynthetic apparatus.

KEYWORDS: **Antioxidant enzymes, Herbicides, Oxidative stress, Photosynthesis, Phytotoxicity.**

RESUMO

O controle de plantas daninhas com glifosato produz danos em plantações de *Eucalyptus camaldulensis*, embora os mecanismos fisiológicos envolvidos não tenham sido completamente elucidados. Este trabalho teve como objetivo avaliar as respostas fisiológicas de *E. camaldulensis* à deriva simulada do glifosato. Os ensaios foram realizados em estufa, com mudas de *E. camaldulensis* clone117 de quatro meses. A deriva do herbicida foi simulada aplicando doses de 0; 43,2; 86,4; 172,8 e 345,6 g a.e. ha^{-1} glifosato. Vinte e três dias após a aplicação foram medidas as trocas gasosas e a fluorescência da clorofila a. Também foram quantificadas a atividade da Rubisco e variáveis indicadoras de estresse oxidativo. O glifosato diminuiu a assimilação fotossintética do carbono, aumentou o quenching não-fotoquímico, induziu o fechamento estomático e aumentou a fotoinibição. Ele também diminuiu a atividade da Rubisco e aumentou a fotorrespiração. O herbicida produziu estresse oxidativo e aumentou as atividades das enzimas catalase, ascorbato peroxidase e superóxido dismutase, envolvidas na desintoxicação de espécies reativas de oxigênio. Conclui-se que os efeitos deletérios do glifosato na assimilação de CO_2 em *E. camaldulensis* são devidos a efeitos estomáticos e não estomáticos. A diminuição da atividade da Rubisco, o aumento da fotorrespiração e fotoinibição destacam-se entre os efeitos não estomáticos. O aumento da atividade do sistema antioxidante é insuficiente para compensar a produção de H_2O_2 na fotorrespiração, que danifica o aparato fotossintético.

PALAVRAS-CHAVE: **Enzimas antioxidantes, Herbicidas, Estresse oxidative, Fotossíntese, Fitotoxicidade.**

INTRODUCTION

Eucalyptus plantations occupy approximately 20 million hectares worldwide and have great economic importance (CERVEIRA JUNIOR et al., 2020). During the first two years of establishment of eucalyptus plantations, weed control is a relevant practice (SANTOS JUNIOR et al., 2015). Glyphosate stands out among weed control methods; it is a non-selective broad-spectrum herbicide (TUFFI SANTOS et al., 2007). Its mechanism of action implies the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), involved in the synthesis of aromatic amino acids (CRUZ et al., 2016). However, the herbicide drift is quite frequent; it reaches tree leaves producing some leaf death and reduced stand growth (TUFFI SANTOS et al., 2011). The physiological processes involved in these responses are not clear.

Although the mechanism of action of glyphosate is well known, a deficiency of aromatic amino acids was not observed after the application of the herbicide in some species, even though they presented the typical deleterious effects (SERRA et al., 2013). Therefore, glyphosate could affect other physiological processes. In some species, glyphosate inhibited photosynthesis with reported stomatal closure and a decrease in the concentration of photosynthetic pigments (GOMES et al., 2016).

Chlorophyll fluorescence analysis is a sensitive and non-destructive method that allows determining damage in the photosynthetic apparatus. It has been used to assess alterations in the photochemical stage of photosynthesis under different environmental stresses (SOUSA et al., 2014). Gomes et al. (2017) used this method to study the effect of glyphosate on photosynthesis in *Salix miyabeana*, proving that the herbicide decreases the potential quantum yield of Photosystem II (PSII). In *Lupinus albus*, glyphosate reduced 26% of the activity of the enzyme Ribulose 1,5-biphosphate carboxylase oxygenase (Rubisco), producing an important decrease in CO_2 fixation (DE MARÍA et al., 2006).

Glyphosate might induce the accumulation of reactive oxygen species (ROS) that produces lipid peroxidation, cell membrane damage, and degradation of proteins and chlorophylls (MITEVA et al., 2010). It has been suggested that tolerance to glyphosate might be related to the capacity of activating antioxidant systems to detoxify such ROS (MAROLI et al., 2015).

This work aimed to evaluate the effects of different glyphosate doses on the physiological responses of *Eucalyptus camaldulensis* (Dehnh.). For this purpose, we hypothesized that, according to applied dose, glyphosate induces damages in the photochemistry of PSII by increasing photo-oxidative stress, decreasing the net assimilation rate.

MATERIAL AND METHODS

Plant material and experimental approach

All the tests were performed under greenhouse conditions in the experimental field of Universidad Nacional de Santiago del Estero (27°45'S, 64°18'W), Argentina. Four-month-old *E. camaldulensis* clone117 seedlings were used. The 40 cm tall seedlings were planted in 10 L pots containing loam soil. Pots were watered daily to keep the soil moisture close to the field capacity.

Doses of 0; 43,2; 86,4; 172,8 and 345,6 g a.e. ha^{-1} glyphosate were applied, corresponding to 0, 3, 6, 12, and 24% of 1440 g a.e. ha^{-1} glyphosate potassium salt (N-phosphonomethyl glycine) produced by Monsanto Argentina under the trademark Roundup® Full II). The herbicide was applied so that it did not reach the upper third of the seedlings with a backpack sprayer equipped with a handheld boom consisting of two flat fan nozzles TT110.02, spaced 0,5 m apart, 250 kPa pressure and water volume of 200 L ha^{-1} (TUFFI SANTOS et al., 2007). During the application, the air temperature was 22 °C and the relative air humidity was 60%.

The greenhouse presented the following environmental conditions: minimum temperature 16 °C, maximum temperature 29 °C, average relative air humidity 52%, and maximum photosynthetic photon flux density (PPFD) 1500 µmol m^{-2} s^{-1}.

Twenty-three days after the application of the herbicide, gas exchange and the chlorophyll a fluorescence were measured, and samples of leaves for chemical determinations were collected.

Gas exchange and chlorophyll a fluorescence measurement

Gas exchange and chlorophyll a fluorescence were measured using an infrared gas analyzer (IRGA LI 6400 XT, LICOR, USA) with an attached leaf chamber fluorometer (LI-6400-40, LICOR, USA). During measurements, the conditions inside the IRGA chamber were set to PPFD 1000 µmol m^{-2} s^{-1}, CO_2 partial pressure 38 Pa, water vapor

pressure deficit 1,2 ± 0,5 kPa, and average air temperature 25 °C. The measurements were performed on the first leave completely expanded between 9:00 a.m. and 11:00 a.m.

Simultaneously with gas exchange measurements, chlorophyll a fluorescence was measured using the saturation pulse technique (SCHREIBER et al., 1994). Measurements were performed on dark-adapted leaves for 30 min, and F_o and F_m (minimum and maximum fluorescence of dark-adapted leaves, respectively) values were registered. Subsequently, leaves were exposed to actinic light (1000 µmol m^{-2} s^{-1}) for at least 30 min. The intensity and length of saturation pulses were 8000 µmol m^{-2} s^{-1} and 0,7 s, respectively. Thus, the maximum quantum yield of PSII (F_v/F_m) and the non-photochemical quenching (NPQ) were calculated according to Maxwell & Johnson (2000). The photorespiration rate was calculated according to Valentini et al. (1995).

Determination of membrane damage, lipid peroxidation and H_2O_2 concentration

To estimate the damage to cell membranes, electrolyte leakage was measured using the technique proposed by Silva et al. (2019).

Lipid peroxidation was quantified through the formation of malondialdehyde (MDA) according to the technique described by Cakmak & Horst (1991). An extinction coefficient of 155 mM^{-1} cm^{-1} was used, and results were expressed as nmol MDA g^{-1} FM.

The concentration of H_2O_2 was determined according to the technique described by Alexieva et al. (2001), and expressed in µmol g^{-1} FM.

Determination of enzymatic activities

The total soluble proteins of leaves were extracted homogenizing 0,2 g of plant material in 100 mM K-phosphate buffer, pH 7, containing 1 mM EDTA, and 1 mM ascorbate. The homogenate was centrifuged at 15000 g for 15 min, and the obtained supernatant was used to perform enzymatic determinations. The concentration of total soluble proteins was quantified as described by Bradford (1976), using bovine serum albumin as standard.

Rubisco activity (EC 4.1.1.39) was measured with the method described by Reid et al. (1997), determining the oxidation rate of NADH at 340 nm.

Catalase activity (CAT, E.C. 1.11.16) was determined according to the technique proposed by Havir & McHale (1987), following the oxidation of H_2O_2, at 240 nm and 30 °C and monitoring the absorbance over 300 s. The activity was calculated using the molar extinction coefficient of H_2O_2 (36 M^{-1} cm^{-1}).

Ascorbate peroxidase activity (APX, E.C. 1.11.1.11) was determined following ascorbate oxidation (NAKANO & ASADA 1981).

Superoxide dismutase activity (SOD, E.C. 1.15.1.1) was determined from the inhibition of the blue formazane production, by the nitro blue tetrazolium photoreduction (GIANNOPOLITIS & RIES, 1977).

Experimental design and statistical analysis

The experiments were arranged in a completely randomized design with 10 replicates. The experimental unit consisted of a pot containing a eucalyptus seedling. Results were analyzed with ANOVA and Tukey´s test ($P < 0.05$).

RESULTS AND DISCUSSION

Photosynthetic carbon assimilation (*A*) was very sensitive to glyphosate; we detected a decrease of 20-80% in plants treated with 43,2 and 345,6 g a.e. ha^{-1} glyphosate, respectively, against control (Figure 1A). In contrast, stomatal conductance (g_s) was not affected by 43,2 g a.e. ha^{-1} glyphosate (Figure 1B); a gradual decrease was observed from the dose of 86,4 6 g a.e. ha^{-1} glyphosate. Higher doses of the herbicide produced an important decrease in g_s; with the dose of 345,6 g a.e. ha^{-1} glyphosate, g_s was 61% lower than in plants without treatment. This result indicates that in the dose of 43,2 g a.e. ha^{-1} glyphosate, photosynthesis was not limited by stomatal closure.

The decrease in *A* was accompanied by the increase in NPQ (Figure 1C), which is an important photoprotective mechanism that allows dissipating the excess of energy absorbed by the PSII light-harvesting complexes (AVILA, 2020).

The F_v/F_m ratio was not affected by doses up to 172,8 g a.e. ha^{-1} glyphosate; however, the dose of 345,6 g a.e. ha^{-1} glyphosate produced a significant decrease of 21% against control (Figure 2A). The relationship F_v/F_m is an indicator of PSII integrity. In healthy plants, F_v/F_m is approximately 0,83; a decrease in the values of this variable indicates photoinhibition (WALTER et al., 2003).

The significant decrease in photosynthetic rate observed in doses of 172,8 g a.e. ha^{-1} and 345,6 g a.e. ha^{-1} glyphosate was accompanied by a decrease in Rubisco

activity of 10% and 25% against control, respectively (Figure 2B).

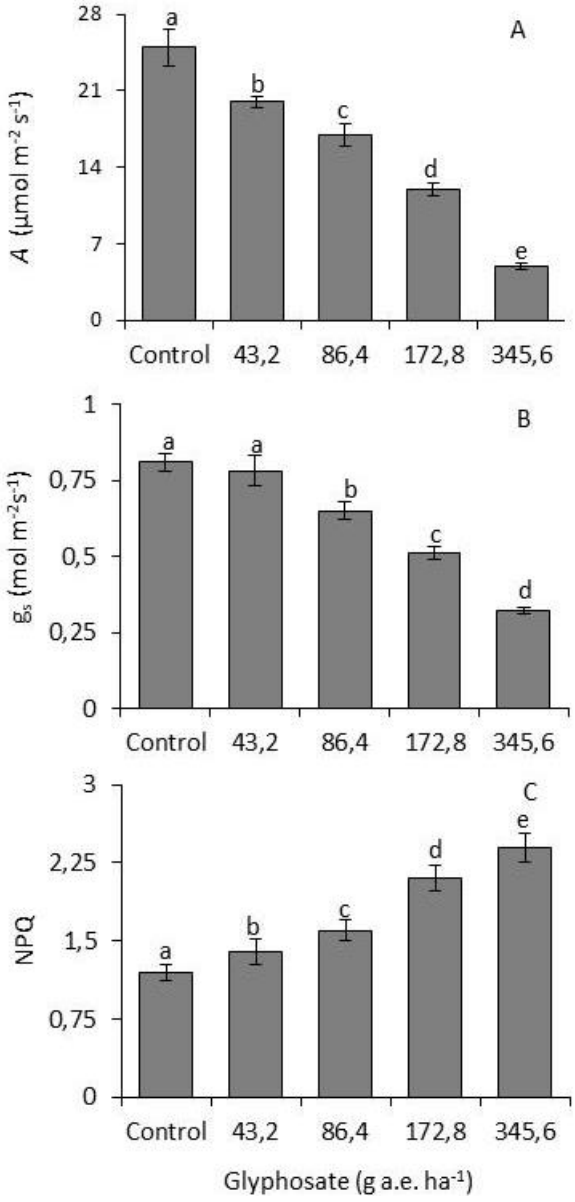

Figure 1. Carbon photosynthetic assimilation, *A* (A), stomatal conductance, g_s (B), and non-photochemical quenching, NPQ (C) in *Eucalyptus camaldulensis* seedlings under simulated glyphosate drift. Vertical bars represent the standard deviation of the mean (n = 10). Different letters denote significant differences at level P < 0.05, according to Tukey test.

The effects of glyphosate on photosynthesis vary in different species and, within the same species, in different genotypes. In *Solanum lycopersicum*, doses of up to 30 mg kg^{-1} glyphosate did not affect *A* or the variables of chlorophyll a fluorescence. In this species, damages generated by glyphosate were associated with an increase in g_s that produced a significant decrease in water use efficiency (SOARES et al., 2020). In *Arachis hypogaea*, 720 g ha^{-1} glyphosate significantly reduced *A*, which is a non-stomatal effect because the intercellular concentration of CO_2 remained constant (RADWAN & FAYEZ, 2016). In *Olea europaea*, glyphosate did not affect net photosynthesis rates, g_s, or the F_v/F_m ratio (CAÑERO et al., 2011).

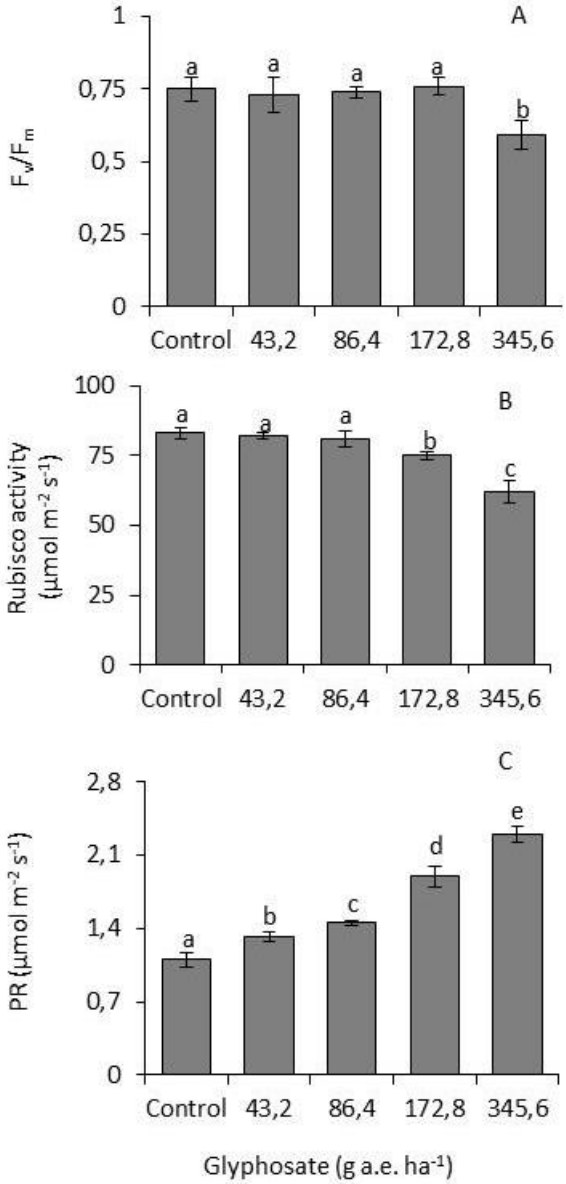

Figure 2. Maximum quantum yield of PSII, F_v/F_m (A), Rubisco activity (B), and photorespiration and PR (C) in *Eucalyptus camaldulensis* seedlings under simulated glyphosate drift. Vertical bars represent the standard deviation of the mean (n = 10). Different letters denote significant differences at level P < 0.05, according to Tukey test.

Cerveira Junior et al. (2020) studied the response to glyphosate in *Eucalyptus x urograndis* clones I140 and GG100 seedlings. They observed that clone I140 was more sensitive to the herbicide than clone GG100, manifested in higher values of *A* and g_s. However, there were no alterations in F_v/F_m in the tested doses, which were within the range 0-180 g a.e. ha^{-1} glyphosate. These authors suggested that the inhibition in the growth of the Eucalyptus clones more sensitive to glyphosate is due to stomatal closure rather than to alterations in the photochemical stage of photosynthesis.

In *E. x urograndis* clone C219, the F_v/F_m ratio was not affected either by glyphosate in doses of 0-720 g a.e. ha^{-1} (CARVALHO et al. 2016). In *E. grandis* clones CLR383 and CLR384, a dose of 86,4 g a.e. ha^{-1} glyphosate produced a decrease of 27% and 32% in A against control, respectively, as well as degeneration of epidermal and parenchymatous cells (SANTOS et al., 2019).

All the tested doses of glyphosate increased photorespiration in *E. camaldulensis* (Figure 2C). This response agrees with the one observed in *Arabidopsis thaliana*, in which glyphosate increased guaiacol oxidase activity, one of the most important enzymes involved in photorespiration (DE FREITAS-SILVA et al., 2017). Vivancos et al. (2011) studied the proteome and redox profiling of glyphosate-sensitive and glyphosate-tolerant soybean cultivars. They obtained evidence of an increase in photorespiration in the glyphosate-tolerant cultivar in the presence of the herbicide.

Photorespiration could contribute to the protection of the photosynthetic apparatus, preventing photoinhibition up to the dose of 172,8 g a.e. ha^{-1} glyphosate, range in which F_v/F_m remained constant. It has been demonstrated that this metabolic process consumes reducing equivalents, protecting the photosynthetic apparatus from photooxidation under stress conditions (LIMA NETO et al., 2017).

Membrane damage was estimated from electrolyte linkage, and it was evident from 172,8 g a.e. ha^{-1} glyphosate (Figure 3A). A similar trend was observed in the concentration of MDA, the product of lipid peroxidation that increased 80% against control in the highest dose of glyphosate (Figure 3B).

The maintenance of selective membrane permeability, observed in the doses of 43,2 g a.e. ha^{-1} and 86,4 g a.e. ha^{-1} glyphosate, coincided with the maintenance of low concentrations of H_2O_2 (Figure 3C). The concentration of such ROS increased from 172,8 g a.e. ha^{-1} glyphosate, reaching its highest values in the dose of 345,6 g a.e. ha^{-1} glyphosate. High concentrations of ROS, such as H_2O_2, superoxide radical ($O_2^{\bullet-}$), and hydroxyl radical ($HO^{\bullet}$), might be produced under stress conditions (CORPAS et al., 2017).

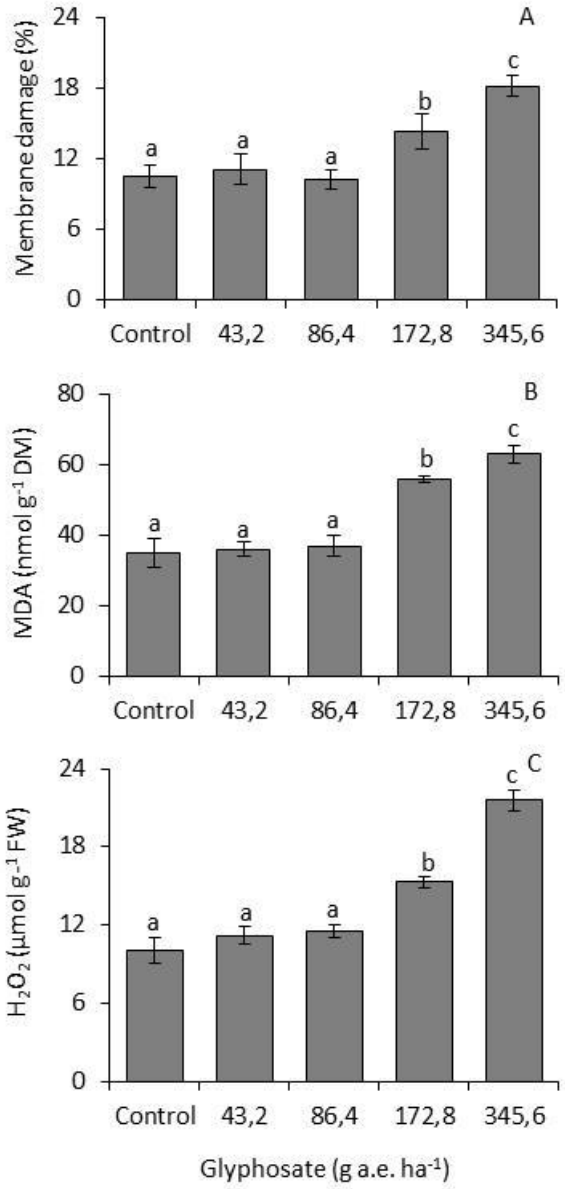

Figure 3. Membrane damage (A), malondialdehyde (MDA) content (B) and hydrogen peroxide (H_2O_2) content (C), in leaves of *Eucalyptus camaldulensis* seedlings under simulated glyphosate drift. Vertical bars represent the standard deviation of the mean (n = 10). Different letters denote significant differences at level $P < 0.05$, according to Tukey test.

In high concentrations, ROS might produce degradation of lipids, protein, and DNA, generating in extreme cases irreversible changes in metabolism (GOMES et al., 2014). Photorespiration is one of the main sources of H_2O_2 in leaves (ZIOTTI et al., 2019); in this

work, the accumulation of H_2O_2 coincided with high photorespiratory rates.

In response to the stress generated by glyphosate, *E. camaldulensis* increased the activity of the antioxidant enzymes CAT, APX and SOD (Figure 4 A, B, C). However, the antioxidant system could not metabolize the produced H_2O_2 because the concentration of this ROS increased from the dose of 172,8 g a.e. ha^{-1} glyphosate. In the case of the dose of 345,6 g a.e. ha^{-1} glyphosate, the increase in the concentration of H_2O_2 could also be due to the increase in SOD activity. This enzyme acts on $O_2^{\bullet-}$ producing H_2O_2 and O_2.

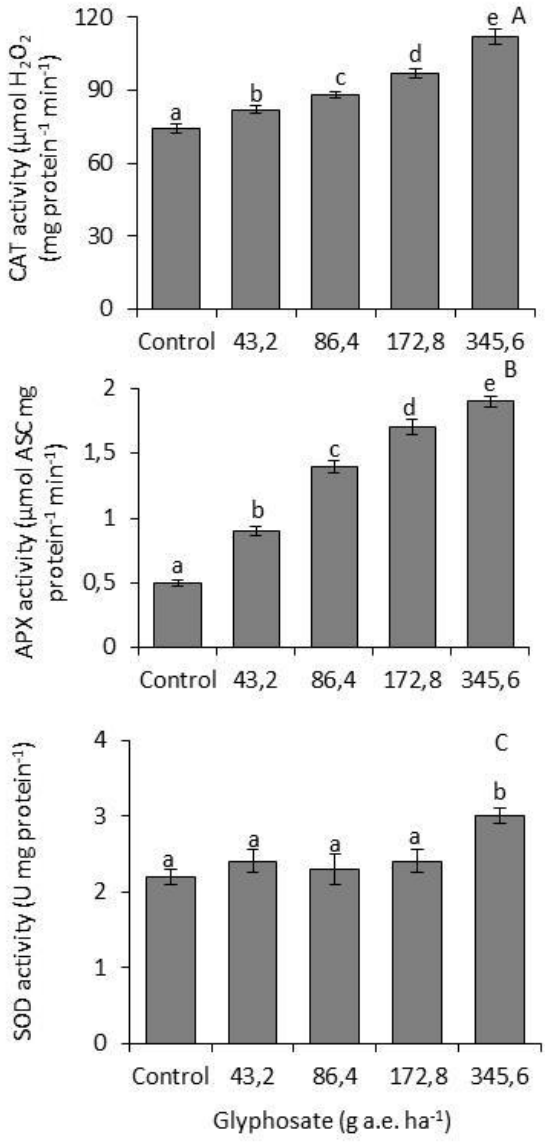

Figure 4. Catalase (CAT) activity (A), ascorbate peroxidase (APX) activity (B) and superoxide dismutase (SOD) activity (C) in leaves of *Eucalyptus camaldulensis* seedlings under simulated glyphosate drift. Vertical bars represent the standard deviation of the mean (n = 10). Different letters denote significant differences at level $P < 0.05$, according to Tukey test.

The oxidative stress generated by glyphosate on *E. camaldulensis* was similar to the response observed in *Salix miyabeana* (GOMES et al., 2017) and *Solanum lycopersicum* (SOARES et al., 2019). In these species, glyphosate increased the concentration of H_2O_2 and, despite the increase of CAT and APX activities, produced lipid peroxidation. In *Arabidopsis thaliana*, glyphosate increased CAT and APX activities, inhibiting SOD activity, whereas the concentration of H_2O_2 remained constant (DE FREITAS SILVA et al., 2017). There was not oxidative stress in glyphosate-sensitive soybean cultivars, as the plants presented low concentrations of MDA (MOLDES et al., 2008).

CONCLUSIONS

We concluded that glyphosate decreases the assimilatory rate of CO_2 in *E. camaldulensis*. This response is mainly due to stomatal and non-stomatal effects. Rubisco activity, increased photorespiration, and photoinhibition stand out among non-stomatal effects. The increase in the antioxidant system activity is insufficient to compensate for the production of H_2O_2 in photorespiration, which damages the photosynthetic apparatus.

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Recebido em 07-10-2020 *Aceito em 04-11-2020*