

Physiological and anatomical responses of *Eucalyptus camaldulensis* leaves to glyphosate application

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Original Article

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Keywords:

Ecophysiology

Pesticides

Plant anatomy

Palavras-chave:

Ecofisiologia

Agrotóxicos

Anatomia vegetal

Received in
2021/05/28

Accepted on
2021/09/21

Published in
2021/10/11



DOI:
<https://doi.org/10.34062/afs.v8i3.12483>

ABSTRACT: The use of herbicides is a usual practice in *E. camaldulensis* nurseries and plantations. The most widely used herbicide is glyphosate, whose drift damages this plant leaves. Although the mechanism of action of glyphosate is well known, it is not clear which alterations lead to the death of plants. The aim of this work was to assess the physiological and anatomical responses of leaves of *E. camaldulensis* seedlings to glyphosate application. Tests were performed on one-year-old seedlings sprayed with 0, 65 and 130 g a.e. ha⁻¹ glyphosate. Ten days after the application, we measured gas exchange and fluorescence emission of chlorophyll a. We also quantify the concentrations of chlorophyll a, shikimate, carbohydrates, K⁺ and Mg²⁺, and the anatomical parameters of epidermis and mesophyll. Data was contrasted using Fisher's test (p<0.05). We concluded that glyphosate alters the physiology of *E. camaldulensis*, inhibiting photosynthesis, changing the metabolism of carbohydrates and the ionic homeostasis. Tissue disorganization, heavily marked at the level of mesophyll, indicates definite alterations.

Respostas fisiológicas e anatômicas de folhas de *Eucalyptus camaldulensis* à aplicação de glifosato

RESUMO: O uso de herbicidas é uma prática usual em viveiros e plantações de *E. camaldulensis*. O herbicida mais utilizado é o glifosato cuja deriva danifica as folhas destas plantas. Embora o mecanismo de ação do glifosato seja bem conhecido, não está claro quais alterações levam à morte das plantas. O objetivo deste trabalho foi avaliar as respostas fisiológicas e anatômicas de folhas de mudas de *E. camaldulensis* à aplicação de glifosato. Os experimentos foram realizados em mudas de um ano de idade pulverizadas com 0, 65 e 130 g e.a. ha⁻¹ de glifosato. Dez dias após a aplicação, foram medidas as trocas gasosas e a emissão de fluorescência da clorofila a. Também foram quantificadas as concentrações de clorofila a, chiquimato, carboidratos, K⁺ e Mg²⁺, e os parâmetros anatômicos da epiderme e do mesofilo. Os dados foram contrastados usando o teste de Fisher (p <0,05). Em conclusão o glifosato altera a fisiologia de *E. camaldulensis*, inibindo a fotossíntese, mudando o metabolismo dos carboidratos e a homeostase iônica. A desorganização do tecido, fortemente marcada ao nível do mesofilo, indica alterações definidas.

Introduction

Weed management is one of the most relevant forestry practices in eucalyptus nurseries and plantations. It is performed with mechanical or chemical methods, or the combination of both (Cerveira Junior et al. 2020).

The recurrent use of herbicides in forest plantation management can be harmful for the crop itself (De Carvalho et al. 2018). Some studies have demonstrated that the contact of herbicides with the leaves of forest species might cause considerable losses in timber production because of growth decline and plant death (Minogue and Osiecka 2015; Santos et al. 2015). The magnitude of the damage is proportional to the dose recommended for weed control (Batista et al. 2018).

Glyphosate (N-phosphonomethyl glycine) is among the most widely used herbicides (Gomes et al. 2019) because it controls a broad spectrum of weeds. It is systemic, nonresidual, and nonselective. It is easily transported from the leaves to the meristematic tissues and acts inhibiting the 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), responsible for the synthesis of chorismate, an intermediary in the shikimic acid pathway leading to the synthesis of aromatic amino acids (Cruz et al. 2021).

Although the mode of action of glyphosate is well known, the metabolic and anatomical changes consistent with plant death after treatment with the herbicide are not clear. Herbicides might decrease the photosynthetic rate (Khan et al. 2020) and alter the metabolism of carbohydrates (Orcaray et al. 2012). The analysis of chlorophyll a fluorescence constitutes a sensitive and nondestructive method for the assessment of the changes produced in the photochemical stage of photosynthesis. It has been recently used to evaluate the degree of damage of the photosynthetic apparatus under different conditions of environmental stress such as extreme temperatures (Van der Westhuizen et al. 2020), drought (Badr and Brüggemann 2020), salinity (Meloni et al. 2017), and herbicides (Souza et al. 2014).

Glyphosate might alter the mineral composition of plants, although the reported results are contradictory (Gomes et al. 2014). In vegetable tissues, the phosphonate and carboxyl groups of the glyphosate molecule can link to divalent cations, immobilizing them (Zobiolo et al. 2011). There are no studies on the effect of herbicides on the mineral composition of eucalyptus.

The impact of the application of herbicides may be observed in the internal anatomy and the epidermis of leaves. However, there are only a few studies on the description of the microscopic damage in leaves (Freitas-Silva et al. 2020). The use of physiological and anatomical variables constitutes a tool to assess the damage of herbicides on woody species (Lima et al. 2017).

The aim of this work is to evaluate the physiological and anatomical responses to glyphosate application on leaves of *E. camaldulensis* seedlings.

Material and Methods

Vegetal material

Essays were performed on one-year-old *Eucalyptus camaldulensis* seedlings grown in plastic pots containing loam soil fertilized with N-P-K (20:5:20). The pots were placed in a greenhouse and received daily manual irrigation to ensure adequate water availability.

Plants were sprayed with Roundup® Full II (Monsanto Argentina), containing 65% (w/w) glyphosate potassium salt (N-phosphonomethyl glycine) as active element at concentrations of either 0, 65 or 130 g a.e. ha⁻¹. Ten days after the herbicide application, measurements of both fluorescence emission and gas exchange were performed, and samples were collected to conduct chemical determinations.

During the light and dark periods, the average values of air temperature were 25°C and 15°C, respectively. The daily average value of solar irradiance was 1,500 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

Photosynthesis and chlorophyll a concentration

Gas exchange measurements were performed at 8:00 a.m. on the first three fully-developed leaves from the apex. An infrared gas analyzer (IRGA-LCpro⁺ System ADC, BioScientific Ltd.) was used in a closed system at a CO₂ concentration of 380 ppm and 26° C. An artificial light source was applied, with a light intensity of approximately 1500 $\mu\text{mol m}^{-2}\text{s}^{-1}$ (Portela et al. 2019). Carbon photosynthetic assimilation (*A*), stomatal conductance (*g_s*), intercellular CO₂ concentration (*C_i*), and carboxylation efficiency (*A/C_i*) were determined.

Chlorophyll a concentration was quantified on the fourth fully-developed leaf from the apex. Leaf samples (0.2 g) were ground in a mortar with 80 % acetone (v/v); the extract was filtered through glass wool and centrifuged at 15,000 x g for five minutes. The supernatant was collected, and the absorbances were measured at 663, 647, and 470 nm. The concentration of chlorophyll a was calculated according to the Lichtenthaler and Welburn equations (1983).

Chlorophyll a fluorescence

Chlorophyll a fluorescence emission kinetics was measured on the same leaves used for the photosynthesis measurements with a Handy PEA portable fluorimeter (Plant Efficiency Analyzer, Hansatech Instruments Ltd, King's Lynn Norfolk, UK). Measurements were performed at 8:00 a.m.,

after the acclimatization of the leaves in darkness for 30 minutes. Fluorescence emission on leaves was induced by red light (650 nm), with an intensity of 3,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ from a matrix containing three diodes focused on a 4 mm diameter point and registered for one second. The OJIP fluorescence transients obtained for each treatment were analyzed according to JIP test, and the index of total performance (PI_{Total}) was calculated according to Gama et al. (2013) using the Biolizer software (Bioenergetics Laboratory, University of Geneva, Switzerland).

Concentrations of shikimate, carbohydrates, and ions

Shikimate was extracted and quantified according to the Singh and Shaner technique (1998). One hundred milligrams of leaves were homogenized in 30 ml HCl 0.25 N. The extract was centrifuged at 25,000 g for 15 m. A 40 μl aliquot of the supernatant was collected to add 0.5 ml of periodic acid 1 %. After 3 h, 0.5 ml NaOH 1 N and 0.3 ml glycine 0.1 % were incorporated. The solution was vigorously mixed, and the absorbance was measured at 380 nm in a spectrophotometer.

Soluble carbohydrates and starch were quantified following the method described by Portela et al. (2019).

For the analysis of ions, leaves were dried in a forced ventilation oven at 70° C for 48 h. Then, they were ground in Wiley-type mill grinder, and the material was digested in a mixture of $\text{HNO}_3/\text{HClO}_4$. The resulting solutions were diluted in deionized water to determine K^+ concentration with a flame photometer (Corning, Model 400, USA) and Mg^{2+} concentration by atomic absorption spectrometry (GBC, Model 908 AA, USA), following the protocol described by Al-Kahayri (2002).

Anatomy of leaves

Mature and fully developed leaves located from the fourth node to the apex were collected to make a homogeneous set for each treatment and a control sample; they were preserved in Carnoy's solution until the moment of processing the material. Blades were extracted from the set of leaves to perform observations and quantifications of the epidermis and anatomy. For the extraction of epidermis, we performed mesophyll digestion with 50 % sodium hypochlorite, a repeated washing with distilled water, an immersion in 5 % chloral hydrate for 5 m, and the mounting on slides using gelatin-glycerin glue. To obtain the transverse sections of the leaves, mesophyll was dehydrated with ethyl series and embedded in paraffin. Cross-sections with 10 μm thick, obtained using a rotary

microtome, were stained with safranin - fast green, and mounted in Canada balsam (Dizeo, 2000). Microscopic observations were performed using a stereo-microscope and anatomical attributes were measured using a Motic BA 210 image processor. In lower epidermis we observed stomatal density, and in the transverse section of leaves, we determined the palisade parenchyma, spongy parenchyma, and total mesophyll thickness.

Statistical analysis and experimental design

An experimental design completely at random with ten repetitions was used, and the results were analyzed with ANOVA and Fisher's test (Fisher $p < 0.05$).

Results and discussion

Glyphosate produced a sharp reduction in the photosynthetic rate (Figure 1A). The 65 g a.e. ha^{-1} and 130 g a.e. ha^{-1} doses decreased in 33 % and 71 % the net photosynthesis when compared with the control, respectively. Stomatal conductance had a similar behavior (Figure 1B), with reductions of 33 y 67 % in both doses with respect to the control, respectively. The inhibition of CO_2 assimilation after applying glyphosate has been observed in other species, but the inhibition mechanism remains unknown (Gomes et al. 2014). The decrease in the net photosynthesis was partly caused by the stomatal closure because both gas exchange variables had the same response to the glyphosate application. However, in the 65 g ha^{-1} glyphosate dose the intercellular CO_2 concentration did not vary with respect to the control (Figure 1C), indicating that CO_2 did not limit photosynthesis in that dose. In agreement with that result, both doses of the herbicide produced a decrease in carboxylation efficiency (Figure 1D), and thus a nonstomatal inhibition of photosynthesis. Glyphosate may decrease carboxylation efficiency by decreasing the levels of ribulose-1,5-biphosphate, and 3-phosphoglyceric acid (Siehl 1997). In *Lupinus albus*, glyphosate produced inhibition of 26% in the Rubisco activity (De María et al. 2006).

Both doses of glyphosate reduced the chlorophyll a concentration (Figure 1E). This response was observed in other species and was associated to pigment degradation or synthesis inhibition (Huang et al. 2012). The accumulation of reactive oxygen species might be responsible for the degradation of photosynthetic pigments (Radwan and Fayez 2016). Synthesis inhibition in chlorophyll a might be caused by low glycine and glutamate concentrations, necessary for the synthesis of δ -aminolevulinic acid, a precursor of this photosynthetic pigment (Serra et al. 2013).

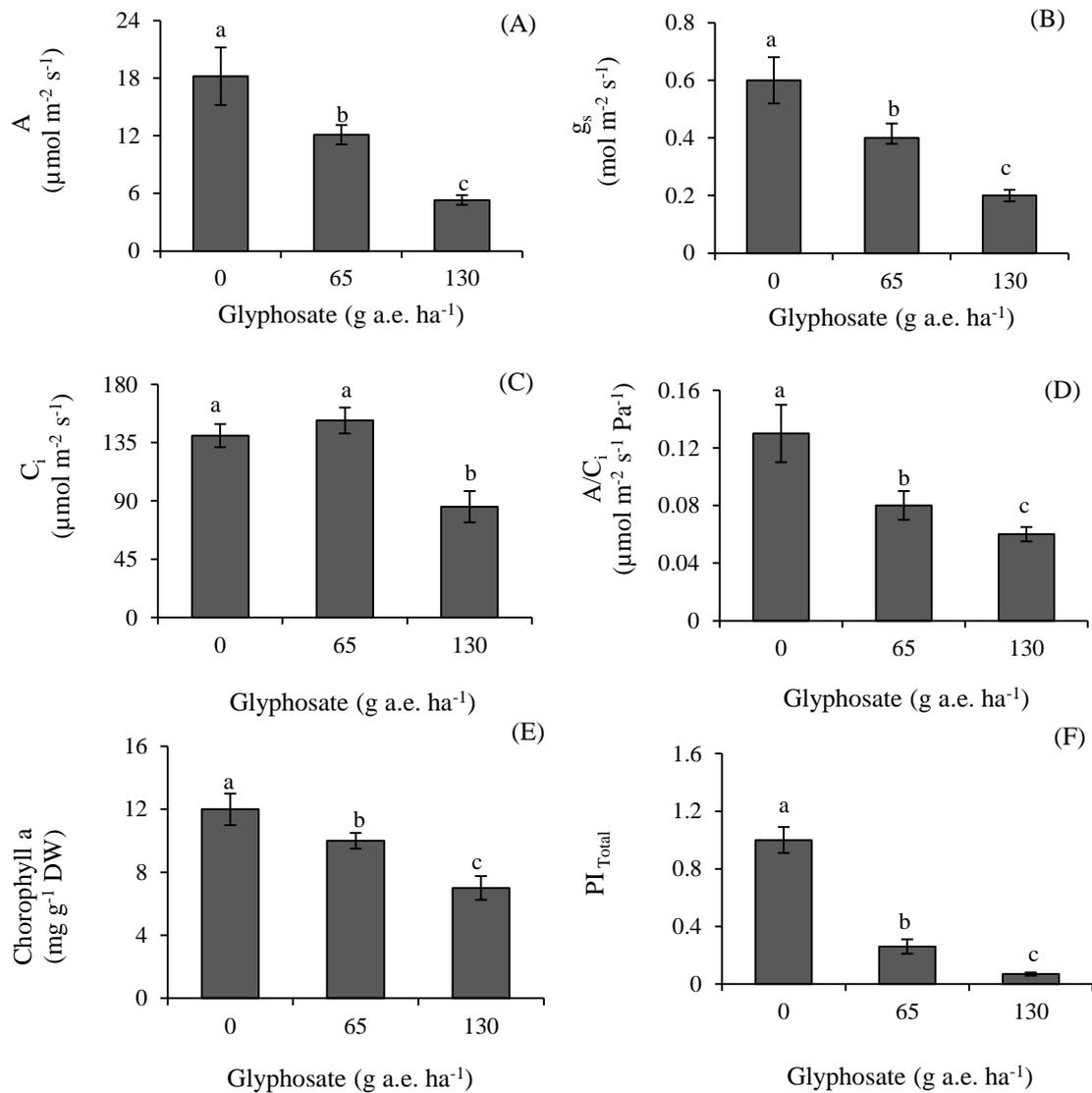


Figure 1. Carbon photosynthetic assimilation (A), stomatal conductance (B), intercellular CO_2 concentration (C), carboxylation efficiency (D), chlorophyll a concentration (E) and index of total performance (F) in *E. camaldulensis* plants submitted to 0, 65 and 130 g a.e. ha^{-1} glyphosate. Vertical bars represent the mean standard deviation. Different letters denote significant differences at level $P < 0.05$ according to Fisher's test.

As a consequence of the impact of the herbicide on the photosynthetic electron transport chain, an important decrease in the total performance index (PI_{Total}) was observed (Figure 1F). PI_{Total} is a fluorescence parameter highly sensitive to environmental stresses that indicates the vitality of the photosynthetic apparatus (Amin et al. 2016). Both doses of glyphosate produced an important decrease in PI_{Total} , which indicates an inhibition in the photochemical stage of photosynthesis. This result was partly caused by the decrease in the content of Chlorophyll a. Gomes et al. (2017) also reported an inhibition in the photochemical stage of photosynthesis in *Salix miyabeana* sprayed with glyphosate because of a

decrease in the concentration of photosynthetic pigments and plastoquinone. In *Glycine max*, glyphosate inhibited the photochemical stage of photosynthesis by decreasing the abundance of the D_1 and D_2 proteins associated to photosystem II (Vivancos et al. 2011). The decrease in PI_{Total} indicates a lower provision of ATP and NADPH for the Calvin cycle (Gama et al. 2013) that coincides with the lowest efficiency in carboxylation calculated from the gas exchange variables.

The obtained results differ from the ones observed in *Phaseolus vulgaris*, in which glyphosate produced an inhibition of photosynthesis because of the stomatal closure whereas the photochemical stage was not altered

(Olesen and Cedergreen 2010). These authors questioned the use of fluorescence variables as markers of stress by herbicides and suggested the use of gas exchange variables.

Shikimate concentration on leaves was very sensitive to glyphosate. In plants treated with both

65 and 130 g a.e. ha⁻¹ glyphosate, shikimate concentrations were 21 and 32 times higher than in the control, respectively (Figure 2A). Glucose and sucrose concentrations also increased in both doses of the herbicide, whereas starch concentration remained constant (Figures 2B, C, and D).

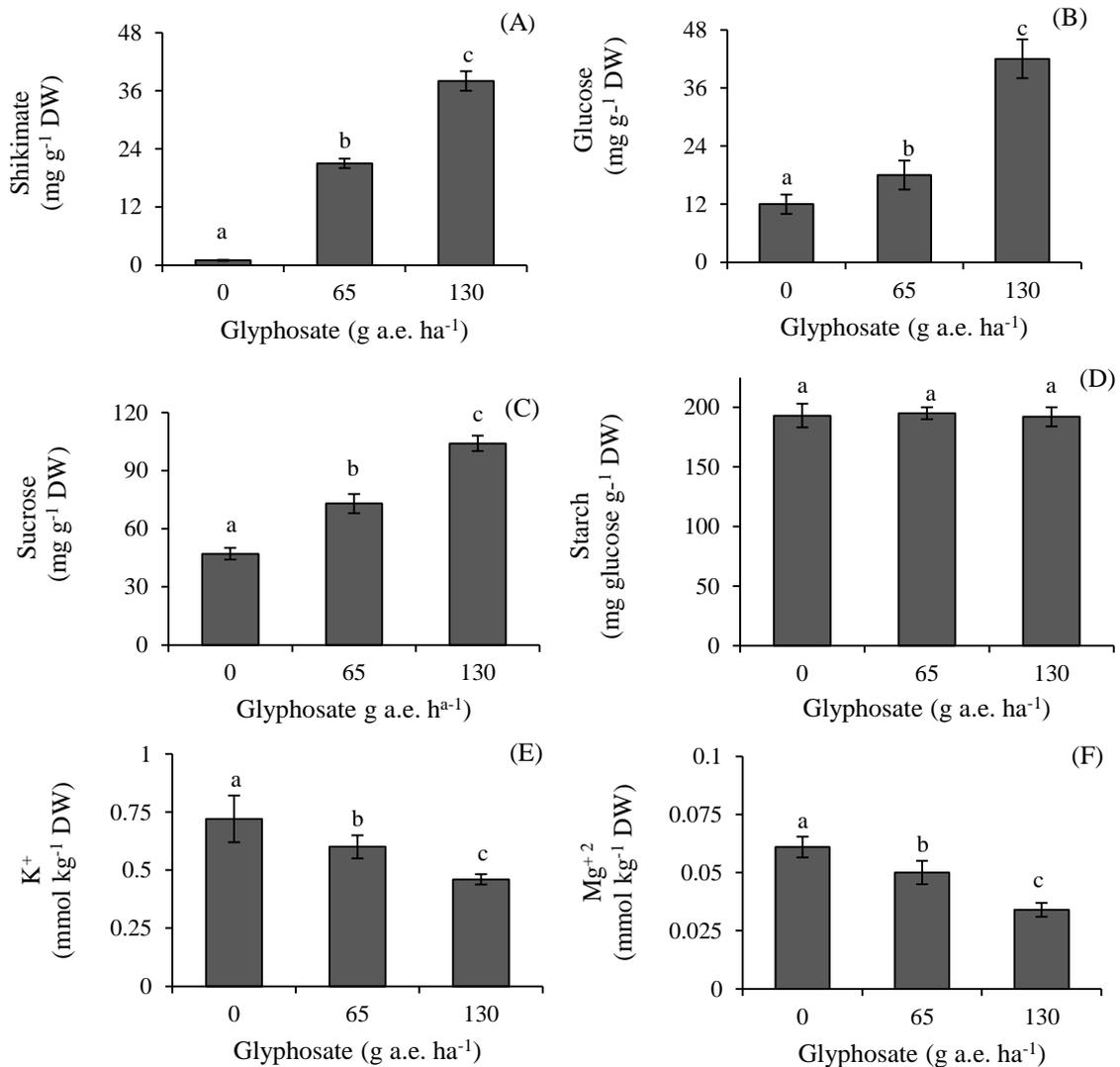


Figure 2. Shikimate (A), glucose (B), sucrose (C), starch (D), potassium (E), and magnesium concentrations (F) in *E. camaldulensis* plants submitted to 0, 65 and 130 g a.e ha⁻¹ glyphosate. Vertical bars represent the mean standard deviation. Different letters denote significant differences at level P < 0.05 according to Fisher's test.

Glyphosate produced a sharp increase in shikimate foliar concentration because of the inhibition of the EPSPS enzyme, representing a measurement of the susceptibility of one species to glyphosate. Thus, a species with low levels of shikimate might tolerate a higher dose of glyphosate (Palma et al. 2019). The high concentration of shikimate indicated the great susceptibility of *E. camaldulensis* to glyphosate, accompanied by an increase in the glucose and

sucrose concentrations in leaves. Because glyphosate inhibited mainly the growing of tissues in active growing, it might produce a decrease in the demand of photoassimilates and thus an accumulation of carbohydrates in leaves. Yannicari et al. (2012) suggested that the stomatal closure and the inhibition in CO₂ fixation observed after the application of glyphosate might be due to the accumulation of the final products of photosynthesis.

Glyphosate affected the mineral composition of leaves. In plants treated with both 65 and 130 g a.e. ha⁻¹ glyphosate, K⁺ concentration was reduced in 17 and 37 % with respect to the control, respectively (Figure 2E). A similar trend was observed in Mg²⁺ concentration (Figure 2F). The effect of glyphosate on mineral nutrition has not been deeply studied, and there are contradictory results (Gomes et al. 2014). Glyphosate did not produce any changes in the mineral composition of glyphosate-resistant soybean cultivars (Zobiolo et al. 2011). However, in glyphosate-sensitive soybean cultivars the absorption of macronutrients decreased, producing mineral deficiencies (Cakmak et al. 2009). Zobiolo et al. (2012) also reported a significant decrease in the concentrations of micro- and macronutrients in soybean leaves treated with glyphosate. These plants had lower absorption and translocation of K⁺ and Mg²⁺. The mineral

deficiencies produced by glyphosate have also been related with the inhibition in root growing (Zobiolo et al. 2012). The decrease in Mg²⁺ and K⁺ concentrations might also be related with the reduction in chlorophyll concentrations and in the stomatal conductance observed in these trials. In effect, Mg²⁺ participates in the synthesis of the porphyrin ring of chlorophylls whereas K⁺ regulates the stomatal openings and closures (Gomes et al. 2014).

The exposition to glyphosate produced a decrease in stomatal density, as the dose increased (Figure 3A), which agrees with observations presented by Lima et al. (2017) in plants of *Bahuinia variegata* using diuron.

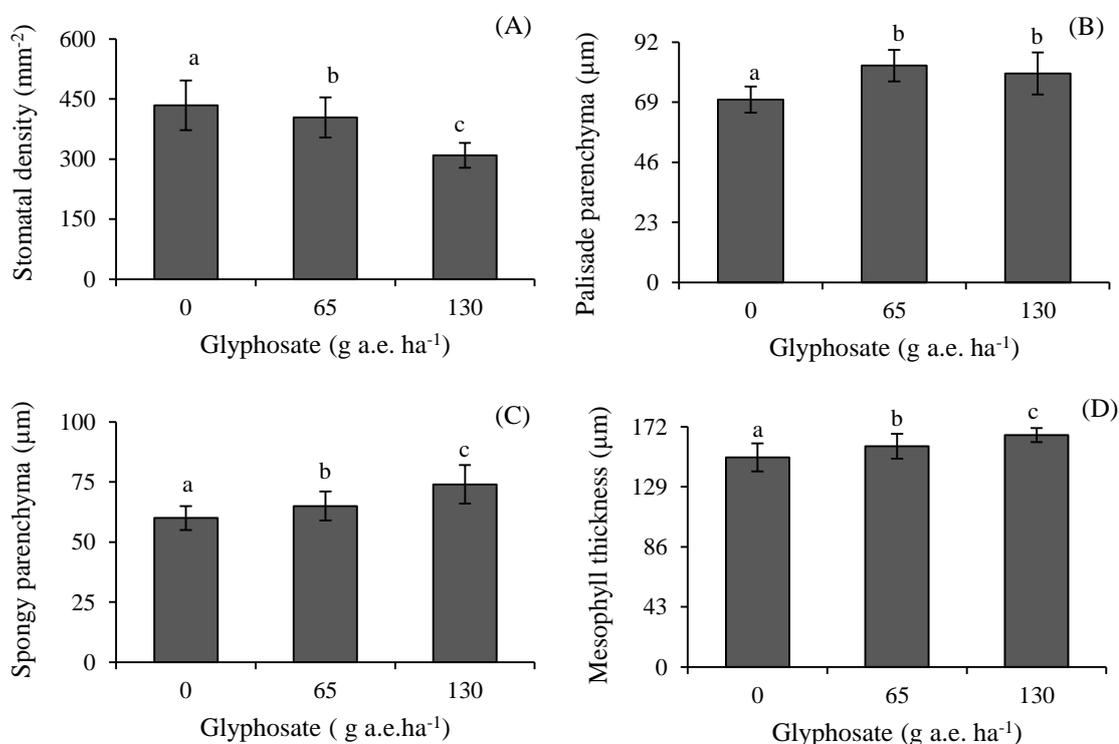


Figure 3. Stomatal density (A), Palisade parenchyma (B), spongy parenchyma (C) and mesophyll thickness (D) in *E. camaldulensis* plants submitted to 0, 65 and 130 g a.e. ha⁻¹ glyphosate. Vertical bars represent the mean standard deviation. Different letters denote significant differences at level P < 0.05 according to Fisher's test.

The thickness of palisade parenchyma, spongy parenchyma and mesophyll, increased significantly as the dose of glyphosate increased (Figures 3B, C and D), in agreement with what was indicated by Tuffi Santos et al. (2008), because of the plasmolysis of some tissues and the hyperplasia manifested essentially in the parenchyma. According to Tuffi Santos et al. (2009), the anatomical responses to the simulation of glyphosate drifting in the foliar anatomy are

associated with the response of the plant as self-protection.

The stomatal inhibition of photosynthesis could have been caused by not only stomatal closure but also by the manifested decrease of stomatal density. The increase of soluble sugars is in accordance with the significant increase of the spongy parenchyma thickness.

Conclusions

Glyphosate alters the physiology of *E. camaldulensis*, inhibiting photosynthesis, changing the metabolism of carbohydrates and the ionic homeostasis. Tissue disorganization, heavily marked at the level of mesophyll, indicates definite alterations.

Acknowledgements

The authors would like to gratefully acknowledge the financial support received from Consejo de Investigaciones Científicas y Tecnológicas, Universidad Nacional de Santiago del Estero (CICyT-UNSE), Argentina.

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