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Original Article

Pouteria torta: a native species of the Brazilian Cerrado as a bioindicator of glyphosate action

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(With 7 Figures)

Abstract

In Brazil, the expansion of agricultural activity and the associated indiscriminate use of herbicides such as glyphosate is directly related to the loss of biodiversity in the Cerrado. The identification of plant species as bioindicators of herbicide action, especially species native to the area, can help in monitoring the impacts of xenobiotics in the remaining Cerrado. Thus, this study was designed to evaluate the possible use of the native Cerrado species *Pouteria torta* as a bioindicator of glyphosate action via changes in physiological performance. At 16 months after sowing, the effect of glyphosate was evaluated by applying the following doses: 0 (control), 25, 50, 100, 200, 400, 800, and 1200 g a.e. ha^{-1}. In response to glyphosate, *P. torta* exhibited reductions in photosynthesis and chloroplastid pigment content, as well as accumulation of shikimic acid and the occurrence of chlorosis and necrosis. These changes demonstrate the high sensitivity of *P. torta* to glyphosate and its potential for use as a bioindicator of this herbicide.

Keywords: phytotoxicity, photosynthetic processes, biomarker, guapeva, herbicide.

Pouteria torta: espécie native do Cerrado Brasileiro como bioindicadora da ação do glyphosate

Resumo

No Brasil, a expansão da atividade agrícola, aliada a utilização indiscriminada de herbicidas como o glyphosate, possui relação direta com a perda da biodiversidade no Cerrado. A identificação de espécies vegetais bioindicadoras da ação de herbicidas, particularmente as nativas do Cerrado, pode auxiliar em processos de monitoramento dos impactos desse xenobiótico nas remanescentes do Cerrado. Assim, este estudo foi projetado para avaliar o possível uso de *Pouteria torta*, espécie nativa do cerrado, como bioindicadora da ação do glyphosate via mudanças na sua performance fisiológica. Após 16 meses de semeadura, o efeito do glyphosate foi avaliado quando aplicadas as seguintes doses: 0 (controle), 25, 50, 100, 200, 400, 800 e 1200 g e. a. ha^{-1}. Em reposta ao glyphosate, as plantas de *P. torta* apresentaram redução na sua performance do processo fotossintético e no conteúdo de pigmentos cloroplastídicos, além do acúmulo de ácido chiquímico e da ocorrência de cloroses e necroses. Essas alterações demonstram a alta sensibilidade de *P. torta* ao glyphosate, o que potencializa a sua utilização como bioindicadora da ação desse herbicida.

Palavras-chaves: fitotoxidade, processos fotossintéticos, biomarcador, guapeva, herbicida.

1. Introduction

The Cerrado is one of the main global hotspots of flora diversity and the second largest of such areas in Brazil (Myers et al., 2000; Silva et al., 2006; Silva, 2010). However, its natural vegetation has been extensively deforested for agricultural use (Françoso et al., 2015; Carvalho et al., 2014). For example, in the central western area of Brazil, there have been losses of 53% of the Cerrado due to intense agricultural development (Beuchle et al., 2015). The expansion and development of Brazilian agriculture is largely associated with intensive use of pesticides, especially herbicides (Procópio et al., 2009; Mancuso et al., 2011). The indiscriminate use of herbicides has had one of the main impacts on the health of organisms (Marris, 2005; Franco-Bernardes et al., 2015). There is consensus about the negative impact of herbicide exposure on non-target organisms, especially by drift processes

(Power et al., 2013; Boutin et al., 2014; Marwitz et al., 2014; Egan et al., 2014). However, there are few studies about efficient methods for monitoring the impact of herbicides on vegetation in the Cerrado (Boutin et al., 2012). The scarcity of such studies is another factor that threatens the biodiversity of this biome.

Biomonitoring has recently attracted attention among methods for evaluating the environmental impact of herbicides (Boutin et al., 2014; Prasad et al., 2015). Reversible and irreversible changes in plant metabolism can be used as bioindicators for the action of xenobiotics (Boutin et al., 2014). Some of the biomarkers used in biomonitoring are the changes in the structure of nucleic acids (Wang et al., 2016), chlorophyll *a* fluorescence, gas exchange (Duke et al., 2003; MacFarlane, 2003), chloroplastid pigment content (Prasad et al., 2015), metabolites, proteins (Schrübbers et al., 2014; Dayan et al., 2015; Hattab et al., 2016), and the occurrence of chlorosis and necrosis in leaves (De-Temmerman et al., 2004).

The glyphosate (N-phosphonomethyl-glycine) causes damage to a wide variety of species (Simmons, 2013), whereas it is systemic and nonselective (Zhao et. al., 2011). This herbicide is used on a large scale and with high frequency (Sihtmäe et al., 2013; Bohn et al., 2014). Glyphosate has a broad spectrum and acts by inhibiting the action of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs), which is considered a key enzyme in the pathway of shikimic acid, a biosynthetic precursor of the aromatic amino acids phenylalanine, tryptophan, and tyrosine (Dayan and Zaccaro, 2012). This herbicide is phloem-mobile and can be rapidly translocated via symplasts to all parts of the plant (Yamada and Castro, 2007; Machado et al., 2010), which enhances its toxic action on all plant tissue.

Considering the ample evidence of the toxic effects of glyphosate to organisms (Franco-Bernardes et al., 2015), it is necessary to study efficient methods of managing of the health of Cerrado areas exposed to this herbicide. Particularly important is the selection of highly responsive and specific biomarkers in plants as potential bioindicators. A bioindicator species is any species which is sensitive to a pollutant, showing alterations at physiological and biochemical levels, in addition to visual symptoms (Nunes and Vidal, 2009).

Pouteria torta (Mart.) Radlk., belonging to the family Sapotaceae, is a species native to the Cerrado. It is an arboreal species with great potential for biomonitoring of herbicide action in the Cerrado since it has wide distribution (Perfeito et al., 2005), which increases its exposure to the drift process of pesticides. This species is popularly known for "guapeva", "curriola", "açá ferro", "abiu do cerrado" and "grão de galo" (Perfeito et al., 2005). It is a lactescent plant, with 8 to 14 m tall, and it has a fluted trunk with a diameter of 30 to 40 cm. It annually produces an abundant fruit load, which is used for human consumption and the peel is used as an anti-dysentery medicine (Perfeito et al., 2005). Its seeds have rapid emergence and also antifungal and insecticide activity (Boleti et al., 2007). Therefore, this study evaluated the hypothesis that *P. torta* is sensitive to herbicide glyphosate, which could be monitored by changes in photosynthetic metabolism. These changes could be used to classify *P. torta* as a potential phytoindicator of glyphosate action in the Cerrado.

2. Material and Methods

2.1. Plant growth and experimental conditions

Fruits were collected from a single specimen of *P. torta* at Fazenda Santo Antonio, Iporá, Brazil (16° 26' 29" S, 51° 7' 11" W). The seeds were obtained and sown in sand. After 60 days, seedlings were transferred to polyethylene pots containing 10 Kg of a substrate consisting of typical dystrophic red oxisol and sand (2:1). This substrate has the following chemical characteristics: pH 6.3 (in water); 0.7 mg dm^{-3} P; 8 mg dm^{-3} K; 240 mg dm^{-3} Ca, 24.3 mg dm^{-3} Mg, 174 mg dm^{-3} H+Al, 11 g kg^{-1} organic matter, and 68% base saturation. Based on this chemical analysis, each pot was fertilized with 1.525 g of urea, 1.175 g of K_2O, 6.9 g of P_2O_5, and 0.4 g of micronutrients (Fritted Traced Elements®, São Paulo, Brazil).

The experiment was conducted in April 2013 in a climatized greenhouse at the Laboratory of Plant Ecophysiology and Productivity of the Federal Institute of Goiás, Rio Verde Campus. The temperature and relative humidity of the greenhouse were monitored during the experimental period using a weather station (WATCH DOG - Weather Station, Spectrum Technologies, Inc., Aurora, IL, USA).

2.2. Treatment application

The effect of glyphosate [Roundup Transorb®, isopropylamine, 480 g L^{-1} acid equivalent (g e. a. ha^{-1}) Monsanto Agricultural São José dos Campos, SP] was evaluated using the following doses: 0 (control), 25, 50, 100, 200, 400, 800, and 1200 g a.e. ha^{-1}. In general crops, glyphosate rates applied vary between 720 and 1700 g e. a. ha^{-1}. The herbicide was applied using a backpack sprayer (Herbicat® Catanduva, Brazil) with constant pressure maintained by compressed CO_2, a boom sprayer with four spray tips, and an extended-range flat spray tip (Teejet XR110/02VP). The working pressure was 5 kgf cm^{-2}, resulting in a spray solution volume of 200 L ha^{-1}.

2.3. Physiological determination

Gas exchange and chlorophyll *a* fluorescence were measured on the second fully expanded pair of leaves in the period between 8 h 00 min and 10 h 30 min. Chlorophyll *a* and *b* and total chlorophyll were measured in the same leaf areas where leaf gas exchange and chlorophyll *a* fluorescence were determined. A total of 10 measurements were performed at 2, 6, 24, 48, 72, 96, 120, 144, 168, and 240 hours after application (HAA) of glyphosate. The measurement times were chosen to detect early physiological changes that may be specific to the effects of glyphosate.

2.3.1. Measurements of gas exchange and chlorophyll a fluorescence

Gas exchange was assayed to determine the photosynthetic rate (*A*, μmol m^{-2} s^{-1}), transpiration rate (*E*, mmol m^{-2} s^{-1}), stomatal conductance (*gs*, mol H_2O m^{-2} s^{-1}), and the ratio of internal CO_2 concentrations to external concentrations (*Ci/Ca*). Measurements were performed using a portable open-system infrared gas analyzer (LI-6400, LI-COR Inc., Lincoln, NE, USA) under an ambient CO_2 concentration and room temperatures of 24 to 26 °C. All of the measurements were conducted under artificial saturating photon irradiance (1,000 μmol m^{-2} s^{-1}) at the leaf level.

The chlorophyll *a* fluorescence parameters were determined using a portable fluorometer with a modulated pulse MINI-PAM (Walz®, Effeltrich, Germany) equipped with a leaf-clip 2030-B (Bilger et al., 1995; Rascher et al., 2000). The leaf was dark-adapted for 30 min and exposed to a weak far-red light pulse (0.5 μmol m^{-2} s^{-1}) for the determination of the initial fluorescence (F_0). A saturating light pulse (0.8 s; 2,400 μmol (photons) m^{-2} s^{-1}) was then applied to estimate the maximum emitted fluorescence (F_m). Using these parameters, the maximum quantum yield of photosystem II (PSII) was calculated (Fv/Fm) (Van Kooten and Snel, 1990).

In light-adapted leaves, the steady-state fluorescence yield (F_s) was measured after a saturating white light pulse (2,400 μmol m^{-2} s^{-1}, 0.8 s) was applied to achieve the light-adapted maximum fluorescence (F_m'). The actinic light was then turned off, and far-red illumination was applied (2 μmol m^{-2} s^{-1}) to measure the light-adapted initial fluorescence (F_0'). Using these parameters, the effective quantum yield of PSII (ΔF/Fm') was calculated (Genty et al., 1989). ΔF/Fm' was used to estimate the apparent electron transport rate (ETR) (Bilger et al., 1995; Laisk and Loreto, 1996), and non-photochemical quenching (NPQ) was calculated according to Bilger and Bjorkman (1990).

2.3.2. Measurements of photosynthetic pigment contents

Chlorophyll *a* and *b* and total chlorophyll were determined using a portable meter (ClorofiLOG1030®, Falker®, Porto Alegre, Brazil) and expressed as the Clorofilog index.

2.3.3. Determination of shikimic acid concentration

Eight leaf discs with a radius of 6 mm (~25 mg) were sampled from the second leaves from the apical meristem of each plant at 2, 6, 24, 48, 72, 120, 168, and 216 HAA to determine the concentration of shikimic acid. The leaf disc samples were kept in liquid N_2 during sampling and then stored at -80 °C until further analysis. Shikimic acid was extracted according to Singh and Shaner (1998) with some modifications. The frozen samples were ground in microtubes containing HCl (0.25 N) at a ratio 1:10 (tissue mass (g): volume of HCl 0.25 N (ml)). The extract was centrifuged at 15,000 × *g* for 25 min at 4 °C. After centrifugation, 30 μL of the supernatant was reacted with 500 μL of periodic acid (1%) for 45 min in a boiling water bath at 37 °C, and then 500 μL of sodium hydroxide (1 N) and 300 μL of glycine (0.1M) were added. The absorbances were measured at 380 nm in a spectrophotometer (UV–VIS Evolution 60S Thermo Fischer Scientific®, Madison, USA). The molar extinction coefficient of 4.76×10^4 M^{-1} cm^{-1} was used to calculate the shikimc acid concentration (Gaitonde and Gordon, 1958), which was expressed in mg g^{-1} of fresh weight (FW).

2.3.4 Analysis of visual symptoms in *P. torta*

To registry of visual symptoms resulting from application of glyphosate in *P. torta*, leaves were photographed at 240 HAA using a 14-megapixel digital camera (Finepix SL 300) with 30x optical zoom and a high-resolution LCD.

2.4. Experimental design

The experiment was carried out in a randomized block experimental design and four replications. The following experimental designs were considered for analysis of variance (ANOVA): (1) an 8 × 10 factorial experiment (doses × evaluation times) for the gas exchange, chlorophyll *a* fluorescence, and photosynthetic pigment content (320 experimental units in total) and (2) an 8 × 8 factorial experiment (doses × evaluation times) for the shikimic acid concentration (256 experimental units). Regression models were fitted to the data using the software SAEG 9.0 (General Statistical Analysis System-Sistema de Análises Estatísticas Gerais, Federal University of Viçosa, Viçosa, Brazil). Graphics were constructed using SigmaPlot V.10 software (SPSS Inc., USA).

3. Results

3.1. Microclimate conditions

During the experimental period, the temperature and relative humidity varied, respectively, between 23 °C and 28 °C and of 61% and 80%. These few variations indicates that there was no overlap of abiotic stress in the plants evaluated, meaning that the results were due to glyphosate action.

3.2. Gas exchange and chlorophyll a fluorescence

The photosynthetic rate (*A*) and stomatal conductance (*gs*) decreased in response to the increasing doses of glyphosate and evaluation times. The glyphosate effects in *A* and *gs* were more pronounced at 240 and 72 HAA, respectively, mostly in plants treatments with the dose of 1,200 g a. e. ha^{-1}, with decreases of 70% in *A* and 75% in *gs* (Figure 1A and 1B). The transpiration rate (*E*) decreased in response to the independent effects of the doses of glyphosate and evaluation times (Figure 2A and 2B), while internal to external CO_2 concentration ratio (*Ci/Ca*) increased solely in response to the doses of glyphosate (Figure 2C and 2D). In particular, decreased of 57% in *E* occurred in the plants treatments with 1,200 g a. e. ha^{-1} compared to their non-herbicide counterparts (Figure 2A and 2B). In response of the increasing doses of glyphosate, *Ci/Ca* reached increase by more 53% at 240 HAA (Figure 2C and 2D).

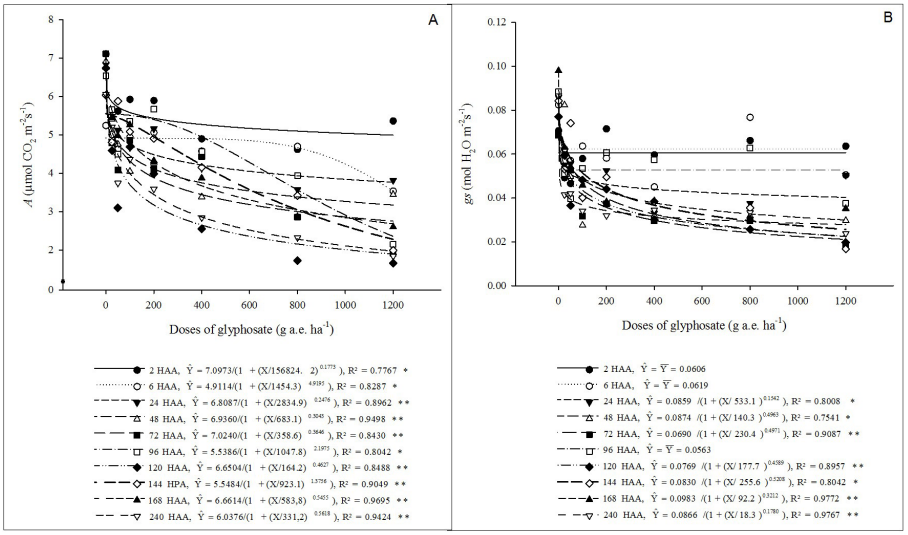

Figure 1. Photosynthetic rate (*A*; A) and stomatal conductance (*gs*; B) determined on the leaves of *Pouteria torta* plants in respose of the interaction between different doses of glyphosate and evaluated and evaluated at ten different times (HAA). Values are averages *n*=4. Significant by factorial analysis (*p ≤ 0.05; **p ≤ 0.01).

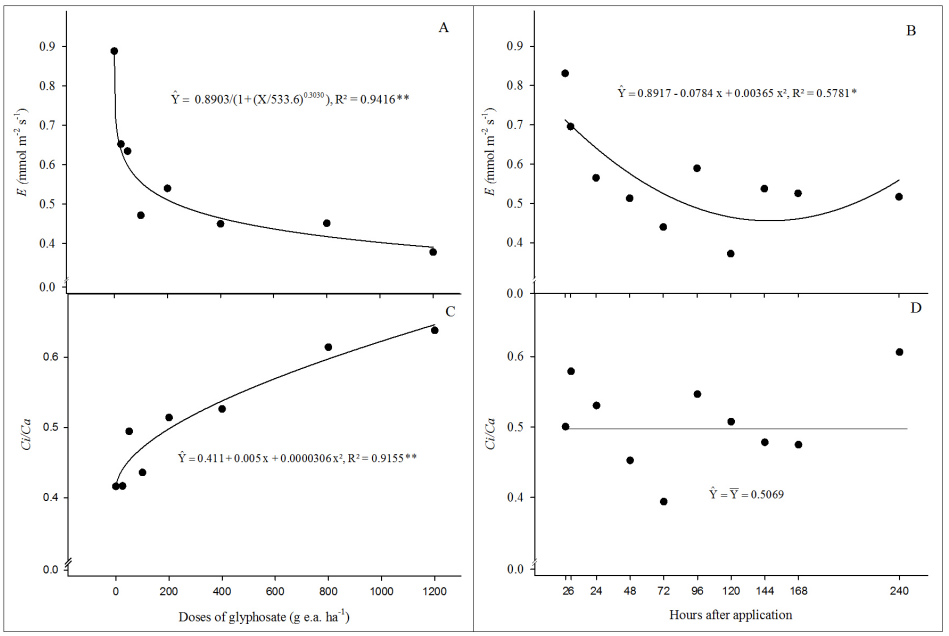

Figure 2. Transpiration rates (*E*; A, B) and internal to external CO_2 concentration ratios (*Ci/Ca*; C, D) determined on the leaves of *Pouteria torta* plants treated with different doses of glyphosate (*n* = 32) and evaluated at ten different times (HAA) (*n* = 40). Significant by factorial analysis (*p ≤ 0.05; **p ≤ 0.01).

The variables of maximum quantum yield of photosystem II (PSII) (*Fv/Fm*), effective quantum yield of PSII (ΔF/Fm'), apparent electron transport rate (ETR) and and non-photochemical quenching (NPQ) exhibited pronounced changes in response to the doses of glyphosate and evaluation times (Figures 3 and 4). The *Fv/Fm* of the control plants remained at approximately 0.63, whereas in the plants treated with glyphosate ocorred a decrease to 0.43, especially from 96 HAA (Figure 3A). There were decrease of 50% in ΔF/Fm' and ETR in response of the increasing doses of glyphosate and HAA (Figure 3B and Figure 4A). The increasing doses of glyphosate promoted

increment in NPQ, but these response was more evident at 240 HAA, reaching an increase of 96% at the highest dose evaluated (Figure 4B).

The contents of the chlorophyll *a*, *b* and total decreased in response to the increasing doses of glyphosate and evaluation times (Figure 5A, 5B, 5C, 5D, 5E and 5F). These effects were more pronounced in plants exposed to dose of 1,200 g a. e. ha^{-1}, with decreases of 23%, de 41% and 27% for chlorophyll *a*, *b* and total respectively (Figure 5A, 5C and 5E).

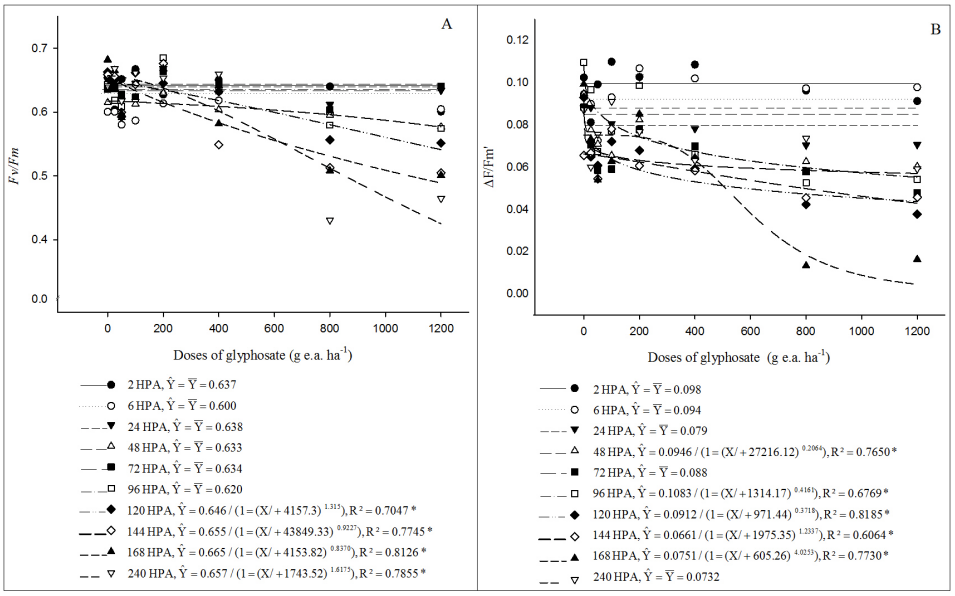

Figure 3. Maximum quantum yield (F_v/F_m; A) and effective quantum yield of the PSII ($\Delta F/F_m'$; B) determined on the leaves of *Pouteria torta* plants in respose of the interaction between different doses of glyphosate and evaluated at ten different times (HAA). Values are averages *n*=4. Significant by factorial analysis (*p ≤ 0.05).

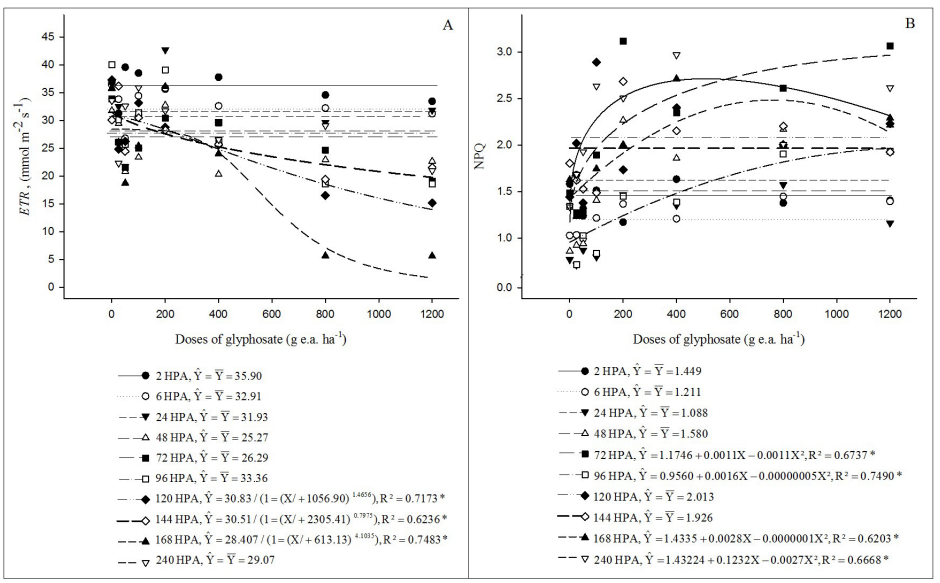

Figure 4. Apparent electron transport rate (*ETR*) (A) and non-photochemical quenching (NPQ) (B) determined on the leaves of *Pouteria torta* plants in respose of the interaction between different doses of glyphosate and evaluated and evaluated at ten different times (HAA). Values are averages *n*=4. Significant by factorial analysis (*p ≤ 0.05).

3.3. Shikimic acid concentration

The shikimic acid concentration varied in response to increasing doses of glyphosate, with increases of 40% in plants exposed to the highest dose (Figure 6A). Regardless of the dose, this increase was observed at 120 HAA (Figure 6B), with accumulation higher than 70% at 216 HAA (Figure 6B).

3.4. Visual symptoms

Plants treated with glyphosate exhibited chlorosis and some necrotic spots, mainly when exposed to the highest doses (Figure 7). The chlorosis occurred in marginal and internerval leaf areas and progressing to the central nerve. Necrotic spots were especially observed in leaf margins and apexes (Figure 7).

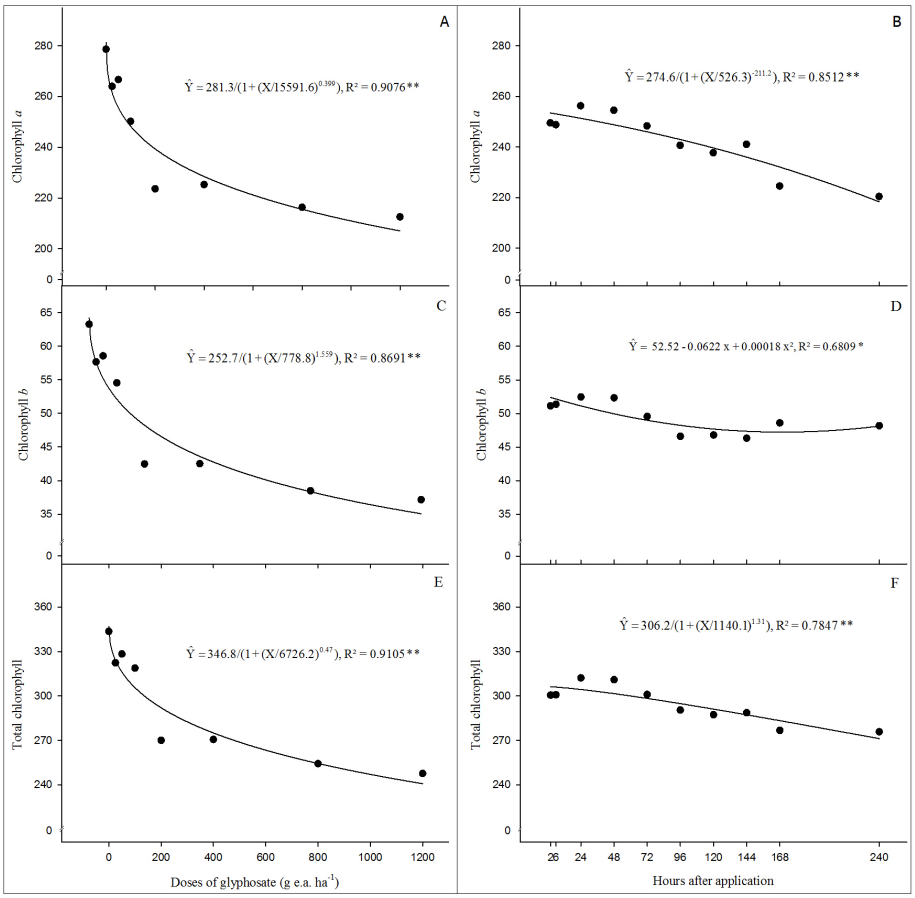

Figure 5. Chlorophyll *a* (A, B), chlorophyll *b* (C, D) and total chorophyll (E, F) determined on the leaves of *Pouteria torta* plants treated with different doses of glyphosate ($n = 32$) and evaluated at ten different times (HAA) ($n = 40$). Significant by factorial analysis (*$p \leq 0.05$; **$p \leq 0.01$).

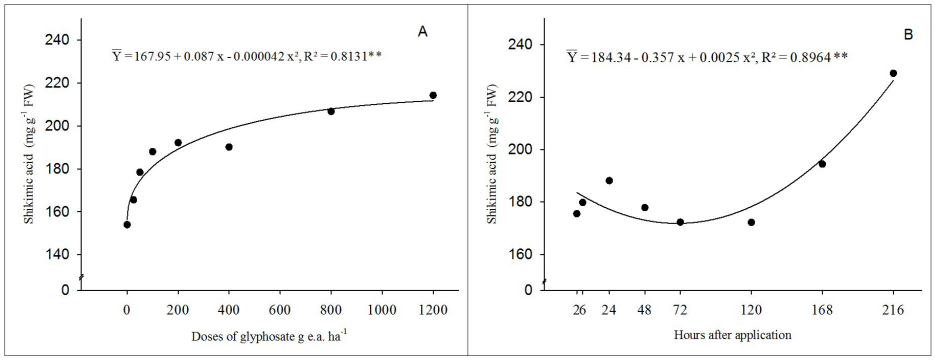

Figure 6. Shikimic acid determined on the leaves of *Pouteria torta* plants treated with different doses of glyphosate (A; $n = 32$) and evaluated at ten different times (HAA) (B; $n = 20$). Significant by factorial analysis (**$p \leq 0.01$).

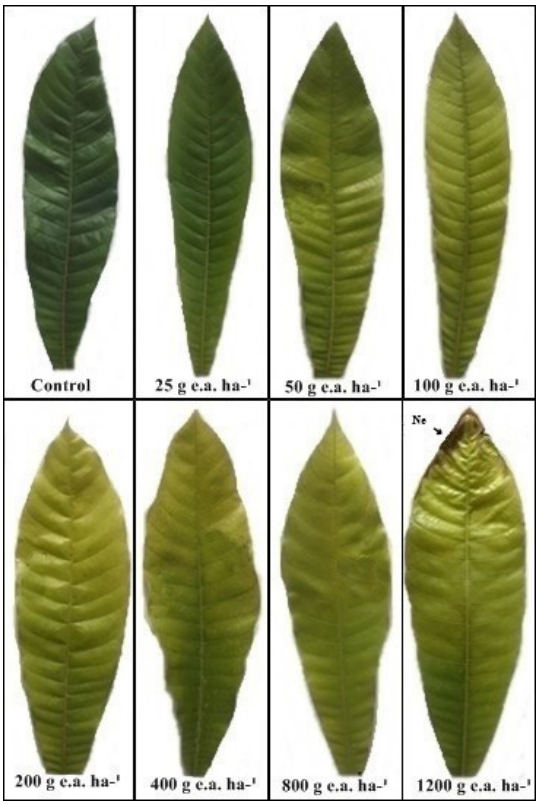

Figure 7. Visual symptoms of toxicity in young leaves of *Pouteria torta* treated with different doses of glyphosate. Ne = necrosis.

4. Discussion

Glyphosate promoted shikimic acid accumulation, which was possibly induced by the reduction in EPSPs activity. This along with foliar phytotoxicity and several changes in physiological processes indicated high sensitivity to glyphosate. These results provide the first evidence of the potential use of *P. torta* for the evaluation of glyphosate's impact in the Cerrado.

The increase in the shikimic acid content is a typical response by susceptible plants to glyphosate (Buehring et al., 2007; Bonini et al., 2009; Reddy et al., 2008; Schrübbers et al., 2014). This herbicide competes with phosphoenolpyruvate (PEP) at the allosteric site of EPSP (Reade and Cobb, 2002; Carvalho et al., 2012). This enzyme catalyzes the transfer of the enolpyruvyl group from PEP to the 5-hydroxyl group of the shikimate-3-phosphate, producing enolpyruvylshikimate-3-phosphate and inorganic phosphate in the shikimic acid pathway (Franz et al., 1997). Therefore, the suppression of the EPSPs activity reduces the efficiency of the shikimate pathway and increases shikimic acid concentrations. The shikimic acid accumulation in *P. torta* supports its potential as a sensitive and specific biomarker of glyphosate action.

Although the photosynthetic process is not the primary target of glyphosate in plants, some studies have shown a reduction in *A* triggered by this herbicide (Duke et al., 2003; Mateos-Naranjo et al., 2009; Orcaray et al., 2010), which supports the results of this study. Yamada and Castro (2007) postulated that the reduced efficiency of gas exchange due to glyphosate is associated with dysregulation of stomatal closure and thus with changes in the opening and closing of stomata. Concurrently with the decrease in *A*, the glyphosate caused a decrease in *gs* and consequently in *E*. Theses results are more than sufficient to indicate that the decrease in the photosynthetic process occurred, at least in part via stomatal limitations. Similar physiological dysfunctions were found in other studies (Olesen and Cedergreen, 2010; Pereira et al., 2010; Yanniccari et al., 2012).

The increase in the ratio between the internal and external concentrations of CO_2 (*Ci/Ca)* results from the increase in CO_2 concentration in the substomatal chamber. In addition to stomatal limitation, it is assumed that biochemical constraints contributed to decreased efficiency in *A* in *P. torta*. Mateos-Naranjo and Perez-Martin (2013) reported that glyphosate decreases the carboxilative efficiency of RuBisCo, possibly by forming reactive oxygen species (Ahsan et al., 2008), which triggers lower efficiency of the Calvin cycle (Watanabe et al., 2013).

A is regarded as the main sink of the absorbed light in chloroplasts, and photooxidative damage can result from the excess energy not used in glyphosate-treated *P. torta* due to drastic decreases in *A*. The activation of the photoprotection mechanism evidenced by the increase in NPQ could contribute to decreasing the energy excess via thermal dissipation. The thermal dissipation is regulated via the xanthophyll cycle and is part of a defense system for avoiding the photoinhibition (Kielak et al., 2011; Marín-Guirao et al., 2013). However, the increased NPQ was not sufficient to mitigate the damage to the photosynthetic apparatus triggered by glyphosate in *P. torta*. This can be supported by the reduction in *Fv/Fm*, which indicates that damage occurred in the structure of the thylakoids, at least in part due to photooxidative stress (Krause and Weis, 1991).

Concurrent decreases of 50% in ΔF/Fm' and ETR of the treated *P. torta* showed that the herbicide affected the process of capture and utilization of the collected energy. These results are consistent with those reported by Mateos-Naranjo et al. (2009), who found severe photochemical limitations in *Spartina densiflora* treated with glyphosate. Thus, due to the high sensitivity of physiological variables to glyphosate, they could potentially be used as biomarkers of the responses of *P. torta* to the action of this herbicide.

Photosynthetic pigments are considered as one of the pillars of photosynthetic performance in plants (Tuffi Santos et al., 2009; Yanniccari et al., 2012; Zobiole et al., 2011). Therefore, decreases in the content of chlorophylls triggered by glyphosate at least partly contributed to the decreases in the efficiency of the photosynthetic apparatus. Glyphosate can affect the content of chloroplastid pigments by inhibiting their biosynthesis and by degradation (Moldes et al., 2008). This herbicide can affect the chlorophyll biosynthesis by its chelating action

with nutrients like nitrogen, magnesium, and manganese, forming slightly soluble metal-glyphosate complexes (Soratto et al., 2004; Zobiole et al., 2011; Mateos-Naranjo and Perez-Martin, 2013), which might have occurred in the *P. torta* plants.

In conclusion, the results of this study showed that glyphosate greatly affects the physiological performance and the shikimic acid concentration in *P. torta*, which especially occurred due to biochemical constraints. These metabolic changes resulted in visible damages in the leaves of *P. torta*, which supports the high sensitivity of this species to glyphosate. The physiological changes and visible symptoms triggered by glyphosate in *P. torta* can be used as sensitive biomarkers to this herbicide. Therefore, this plant species has potential to be used as a phytoindicator of glyphosate action. In addition, the results of this study provide the first evidences about the imminent risk of biodiversity loss in Cerrado remnants by glyphosate, in case of indiscriminate use of this herbicide.

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