Tolerance of Hymenaea stigonocarpa Mart. ex Hayne. to glyphosate

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Abstract

The objective of this work was to evaluate the glyphosate herbicide effect on *Hymenaea stigonocarpa* Mart. ex Hayne seedlings. A randomized block design with 5 replicates was used with an experimental unit composed of one *H. stigonocarpa* plant in 5L pots. The treatments were: 0 (control); 9.6; 240; 480; 960 g a. ha^{-1} of glyphosate. Evaluations were performed 24 hours and 60 days after application. Gas exchange, respiration, photosynthesis, visual, anatomical and histochemical evaluations were carried out with leaves from the middle third being collected, and the growth in relation to the two dates was measured and recorded. The growth analysis showed that the seedlings showed an increase in stem diameter, a decrease in leaf number and an increase in height. There were increases in photosynthetic, electron transport and transpiratory rates, and in the effective yield of photosystem II one day after the glyphosate application. The plants then showed a reduction in transpiratory rate and a reduction in Fv/Fm at 60 days after application. With these studies we can conclude that the *Hymenaea stigonocarpa* species is able to survive after contact with the glyphosate herbicide, with no visual and/or anatomical damage, along with positive increases in growth and physiological characteristics.

Introduction

Glyphosate is one of the most widely used herbicides in the world and has been marketed for over 35 years [1]. It is an herbicide which is widely used to control weeds to eliminate them at an early stage of development [2], acting on sensitive plants by blocking the enzyme activity, interrupting the activity of the plastid enzyme 5-enolpyruvylshikimate-3-phosphate synthesis (EPSPS) [3]. The shikimate route is interrupted by the herbicide and in turn the biosynthesis of the essential phenylalanine, tyrosine and tryptophan aromatic amino acids which are indispensable in protein synthesis, cell division and plant growth, and can lead to plant death [4, 5].

Research with herbicide drift enables evaluating the effects and selection of tolerant or bio-indicator species; phytotoxicity is a current problem and causes serious damage to non-target crop species [6]. One of the visible symptoms is chlorosis, and depending on the degree of intoxication it can lead to inuria. The symptoms depend on several factors such as the species, the development stage, the climate, the dose and the amount of active principles that reach the plant, and may benefit the morphological characteristics of the species [3, 7, 8, 9, 10]. Therefore, the use of herbicide in chemical control needs to be based on strict criteria, taking into account cost, efficiency, as well as environmental and human safety, and must be integrated into weed control [11, 12].

Photosynthesis can be affected by herbicides displaced from the application site by drift [10]. Glyphosate indirectly influences photosynthetic processes [13, 14, 15]. Another important tool that helps in detecting damage caused by contact with herbicides is anatomically evaluating the leaf, which determines uptake by the plant. This contributes to understanding the barrier that each species imposes to its penetration [16, 17]. In addition, micromorphological changes occur before visual damage [9].

The Cerrado is the second largest Brazilian domain and has been changing from the expansion of agricultural frontiers due to the increase in the demand for meat and grains for export, promoting a depletion of natural resources [18]. Such changes make the Cerrado one of the main tropical ecosystems, being a hotspot for the preservation of our planet's biodiversity [19]. Native species start to occupy close environments with agricultural crops due to this change in the biome, in turn becoming subject to the action of herbicides and undergoing unwanted exposure caused by the action of the winds and physical proximity, which has caused damage to the environment [20].

Hymenaea stigonocarpa Mart. Ex Hayne is a large tree naturally occurring in the Cerrado domain. They have leaves with adaxial and abaxial epidermis covered by a very thick cuticle, and the presence of oil glands in a subepidermal

location has also been observed [21]. These anatomical structures are involved in decreasing the effective amount of herbicide absorbed by plants. Glyphosate has little affinity for lipids, therefore epicuticular waxes with large amounts of non-polar compounds constitute a barrier to the penetration of this herbicide [22, 23, 24].

Based on similar results, it is possible to search for new planting technologies for the recovery of degraded areas, either to control weeds in cultivation or even to implement protection belts for existing native forest fragments in agricultural areas [25].

In view of the above, this work aims to evaluate the impact of the glyphosate herbicide on the leaf blade growth, physiology and anatomy of the *H. stigonocarpa*. species (jatoba), as well as to test the plant's tolerance to the herbicide.

Material And Methods

Experimental design and application of treatments

The experiment was carried out in an acclimatized greenhouse (temperature ~ 25.5°C and relative humidity ~ 74%) at the Instituto Federal Goiano, Rio Verde Campus, Brazil. *H. stigonocarpa* seedlings at 8 months were obtained from a commercial nursery, being replanted in 5 L pots, totaling 25 plants. The exsicata de *H. stigonocarpa* plants was deposited in the herbarium of the Rio Verde in Goiano Federal Institute under catalog number 10039.

The physicochemical characteristics of soil (dystrophic Red Oxisol) were: pH 5.12; 1.06 $cmol_c\ dm^{-3}$ Ca; 0.48 $cmol_c\ dm^{-3}$ Mg; 1.6 Ca + Mg, 0.05 $cmol_c\ dm^{-3}$ Al; 2.1 H + Al, 0.33 $cmol_c\ dm^{-3}$ K; and 128 mg dm K; 8.4 mg dm^{-3} S; 2.6 mg dm^{-3} P; and 48% clay, 12% silt and 40% sand. Irrigation was performed daily with a nutrient solution containing ammonium sulfate (100 mg L^{-1}), monoammonium phosphate (150 mg L^{-1}), potassium chloride (110 mg L^{-1}), copper sulfate (58 mg L^{-1}), boric acid (81 mg L^{-1}) and zinc sulfate (173 mg L^{-a}) [26].

The experiment was carried out in a randomized block design in a 5 × 2 factorial scheme with five doses (0 (control), 96, 240, 480, 960 g ha^{-1}) and two evaluation dates (1 and 60 days after application), with five replicates. The experimental unit was a pot containing one *H. courbaril* plant. The plants were then exposed to glyphosate doses (Roundup Transorb®, 480 g ha^{-1} of the acid equivalent) after ~ 60 days of acclimatization.

A control treatment was conducted in parallel without herbicide application, 0 (Control) 96, 240,480, and 960 g ha^{-1}, which represent 10, 25, 50, and 100% of the recommended doses for 480 g ha^{-1} for control of tree species. Application was performed using a backpack sprayer with constant pressure maintained by compressed CO_2, equipped with a bar with four spray tips and a XRTeejet® series nozzle with a XR11002-VP fan. A total of 120 L ha^{-1} of spray solution was applied. The application was carried out at 8:00 am, wind speed at 1 m s^{-1}, average temperature of 18.3°C and the relative humidity was 90%. The pots were transferred to the greenhouse after the treatments were applied.

Visible leaf symptoms

The fully expanded leaves were photographed with a semi-professional camera (Cyber-Shot SONY HX100V) for visual analysis of the leaves which best represented the treatment to be used to make the board [27].

Leaf morphoanatomical characterization

Leaf samples were collected 24 h and 60 days after the application of the herbicide for the morphoanatomical analysis. Leaf samples of 3 cm^2 were collected from the central region of the last fully expanded leaf from all replicates of each treatment of the *H. stigonocarpa* plants. The samples were initially fixed in Karnovsky solution for 24 h [28]. After this period, the plant material was pre-washed in phosphate buffer (0.1 M, pH 7.2) and dehydrated in an increasing ethyl series (30–100%), pre-infiltrated and infiltrated in historesin (Leica, Germany), according to the manufacturer's recommendations.

The samples were then transversely sectioned at 5 µm thick in a rotating microtome (Model 1508 R, Logen Scientific, China) and the sections were stained with toluidine blue - polychromatic staining (0.05% 0.1 M phosphate buffer, pH 6.8). Images were obtained through an Olympus microscope (BX61, Tokyo, Japan) coupled with a DP-72 camera using the bright field option. Morphoanatomical observations of the epidermis were subsequently performed on the adaxial and abaxial surfaces, palisade and spongy parenchymas and mesophyll [29].

Histochemical test

Samples previously fixed in FAA70 and SFF were included in historesin. The sections were stained with ferric chloride for the reaction of phenolic compounds [30] and Lugol for detection of starch grains. The respective controls were simultaneously performed with the histochemical tests according to standard procedure.

Micromorphometric measurements

Micromorphometric measurements were obtained from the records of anatomical and histochemical images captured through an Olympus microscope (BX61, Tokyo, Japan) using the ImageJ software program (Image Processing and Analysis in Java, v. 1.47, USA). Measurements were made in ten observations per repetition 5 for each evaluated structure [31].

Evaluation of chlorophyll a fluorescence and gas exchange

Measurements were taken after 24 h and 60 days after herbicide application. Chlorophyll *a* fluorescence variable was evaluated using a portable LI-6800 infrared gas analyzer (IRGA) (Li-cor, Nebraska, USA). The minimum fluorescence analysis (F_0) was performed before dawn through excitation of the leaf tissues by low intensity modulated red light (003 µmol photons m^{-2} s^{-1}). Next, saturation impulses of approximately 8000 µmol photons m^{-2} s^{-1} were applied for 0.8 s to obtain maximum fluorescence (F_m). From these values it was possible to determine the potential photochemical quantum yield of photosystem II (PSII: $F_v/F_m = [F_m\text{-}F_0]/F_m$). After exposing the leaves to light, minimum fluorescence (F_0) was obtained by: $F_0 = F_0/[(F_m - F_0/F_m) + (F_0/F_m')]$ [32].

Non-photochemical quenching ($qN = [F_m\text{-}F_0] / [F_m\text{-}F_o]$ [33], and non-photochemical extinction coefficient ($NPQ = [F_m\text{-}F_m']/F_m$) were determined. Apparent electron transport rate (ETR) was calculated as ETR = Φ FSII × PAR × 0.5 × 0.84 [34], where PAR represents photosynthetically active radiation (µmol m^{-2} s^{-1}) in leaves, 0.5 is the fraction of excitation energy directed to PSII and 0.84 leaf absorbance [31]. The maximum efficiency of PSII photochemistry in adapted-light leaves ($F_{v'}/F_{m'} = [(F_{m'}-F_{o'})/F_{m'}]$), the quantum yield of the assimilation of CO^2 (ΦCO^2), and the relationship between the (Φ_{PSII}) were calculated according to the methodology proposed by [35]. Gas exchange form in *H. stigonocarpa* plants were measured in the same leaf as the chlorophyll *a* fluorescence. The photosynthetic rate was measured in the same leaf using the LI-6800 system. The photosynthetic rates (*A*, µmol $m^{-2}s^{-1}$), stomatal conductance (*gs*, mol H_2O $m^{-2}s^{-1}$), transpiration rate (*E*, mmol $m^{-2}s^{-1}$) and the ratio between internal and external CO_2 concentration (C_i/C_a) were determined under constant photosynthetic photon flux density (PFFD, 1000 µmol m^{-}

$^{2}s^{-1}$) and CO_2 concentration (400 µmol mol^{-1} air), and environmental temperature (~ 25.5 C) and relative humidity (~ 74%). The evaluations were performed between 8:00 am and 11:00 am.

Morphological analysis

Morphological measurements began after applying glyphosate doses. *H. stigonocarpa* seedlings were measured monthly for plant height (PH, cm) considering the length of the main stem to the apex of the stem, and the stem diameter (SD, mm), which were measured with a millimeter ruler and digital caliper, respectively; the total number of expanded leaves (NL) [35]. Values obtained after 60 days after the application of the glyphosate doses were analyzed.

Statistical analysis

The results were submitted to Shapiro-Wilk's normality tests and Bartlett's test for homogeneous variances at 5% of significance. Next, the data underwent variance analysis (ANOVA) by the *F*-test, being adjusted to regression models at 5% significance level. The models were chosen for simplicity, biological significance and the coefficient of determination. Statistical analyzes were performed using the SISVAR statistical program and graphs were performed using Excel.

Results

Visible leaf symptoms and morphoanatomical characterization

Visible damage to the leaf blade was not observed in any of the treatments (Fig. 1).

The evaluation of anatomical changes showed that the leaf epidermis of *H. stigonocarpa* is made up of isodiametric cells with flat or slightly convex anticline walls and amphi-stomatal leaves on the adaxial and abaxial surfaces. The mesophyll is, composed of one or two layers of palisade parenchyma, four to five layers of spongy parenchyma and secretory channels. The glyphosate doses did not cause changes in *H. stigonocarpa* during the analysis period (Fig. 2).

Histochemical test

The presence of starch grains in the mesophyll, adaxial epidermis and abaxial epidermis of *H. stigonocarpa* leaves was not observed in the histochemical tests, regardless of the glyphosate dose used in the study (Fig. 3).

The presence of phenolic compounds in the leaf cells of *Hymenaea stigonocarpa* was not observed in either the control treatment or in the treatments with glyphosate doses (Fig. 4).

Morphological analysis

When analyzing the relative growth rate for the *H. stigonocarpa* plants, it was possible to verify that the diameter presented a quadratic behavior, and all treatments were better than the control (Fig. 5A), whereas the relative growth of new leaves and the relative growth in height showed negative quadratic behavior (Fig. 5B-C).

The effects of glyphosate doses were not significant in the micromorphometric analyses by the F-test at 5% probability for the thickness of leaf tissues of *H. stigonocarpa* plants. The averages for the evaluated characteristics are presented below (Table 1).

Table 1
Leaf tissue thickness, F-test at 5% probability for *H. stigonocarpa* Mart. ex Hayne. seedlings 60 days after glyphosate application. (AdEp) adaxial epidermis. (AbEp) abaxial epidermis. (PP) palisade parenchyma. (SP) spongy parenchyma. (CutAd) adaxial cuticle. (CutAb) abaxial cuticle

Dose ($g\ ha^{-1}$)	Leaf (µm)	EpAd (µm)	EpAb (µm)	PP (µm)	Sp (µm)	Mesofilo (µm)	CutAd (µm)	CutAb (µm)
0	197,942	18,205	15,966	72,709	89,408	162,538	4,432	4,476
9,6	190,423	15,572	13,880	62,629	92,383	156,323	4,327	4,346
240	187,893	17,859	14,424	61,674	91,780	155,280	4,482	4,48
480	190,650	17,608	15,550	60,023	97,129	156,010	4,566	4,586
960	174,924	18,083	17,003	44,906	94,928	138,994	4,489	4,489
Average	**188,366**	**17,465**	**15,364**	**60,388**	**93,126**	**153,829**	**4,486**	**4,475**

Evaluation of chlorophyll a fluorescence and gas exchange

Physiological analyzes of *H. stigonocarpa seedlings* one day after glyphosate application showed positive quadratic behavior for photosynthetic rate (Fig. 6A), electron transport rate (Fig. 6B), transpiration rate (Fig. 6C) and on the effective quantum yield of photosystem II. (Fig. 6D), except for transpiration rate, where there was an increase only up to dose 240; all glyphosate doses increased the values of the aforementioned characteristics in relation to the control.

The second physiological evaluation on *H. stigonocarpa* seedlings 60 days after application showed that the transpiration rate decreases with higher glyphosate doses (Fig. 7A) and the potential quantum yield of photosystem II showed a quadratic behavior, with the highest dose showing lower values compared to other treatments (Fig. 7B).

Discussion

Glyphosate did not affect the morphology and anatomy of *H. stigonocarpa* leaves, regardless of herbicide doses and evaluation time. There are reports of weeds whose herbicide tolerance mechanism is due to low absorption and/or translocation of the herbicide to the action site [17, 24, 38, 39, 40], which is why it led us to believe that the wax layer or cuticle serves as a physical and hydrophobic barrier that hinders absorption via the leaves [41].

Glyphosate can present different responses in relation to morphological characteristics, being beneficial or inhibiting [42, 43]. In the case of *H. stigonocarpa*, glyphosate promoted stem thickening the diameter, which in turn is one of the characteristics whose analysis enables indicating the ability of a seedling to survive in the field and should be used as one of the best indicators of seedling quality and consequently survival in the field [44, 45]. Stem thickening is an important parameter to generate greater plant resistance and support the weight of leaves and fruits [46]. Species that have morphological characteristics which hinder glyphosate absorption causing the compound to only be absorbed in sub-doses may be related to “beneficial” changes; first, the occurrence of physiological changes such as photosynthetic rate, electron transport, transpiration and on the effective quantum yield of photosystem II stimulate the species to produce more photoassimilates [3]. The increase in these rates positively influences the plants, and this is all related to the degree of absorption; low doses have a hormesis effect as already reported in other forest species, and thus promote a positive effect of stem thickening [47, 48].

Defoliation can probably be related to lower plant height production, a fact which is intensified by the decrease in transpiration rate and potential quantum yield of photosystem II verified at 60 days after glyphosate application. Leaf

abscission has already been related to a reduction in photosynthetic capacity [49, 50], and with an increase in the herbicide dose, the intoxication level increased, causing leaf abscission and consequently a reduction in leaves to perform photosynthesis and is related to the drop in the growth rate of *H. stigonocarpa* seedlings. Defoliation reduces the production of carbohydrates for plants, followed by a high demand for photoassimilates [51]. Thus, something similar is believed to have occurred with *H. stigonocarpa* from the Cerrado, in which the energy demand for plant growth was not met at the highest glyphosate doses.

It is known that the main symptoms of glyphosate appear hours after application and include chlorosis followed by necrosis, and become more accentuated according to the dose of the active ingredient in sensitive plants. However, these symptoms were absent during the analysis period and doses. Anatomical analyzes on leaves are important tools that help in the detection of damage caused by contact with herbicides, as these can determine absorption by the plant [16]. However, for this work, no anatomical damage was observed in the leaf structures evaluated. Glyphosate is rapidly absorbed and transported to meristematic tissues [52], and doses higher than 360 g e.a h^{-1} would be enough to kill the plant 21 days after glyphosate application, which did not occur with *H. stignocarpa* [53]. We can analyze the defense mechanism through anatomical evaluations, which contributes to understanding the barrier that each species imposes to its penetration [16]. In the case of *H. stignocarpa*, it was found that the main protection barrier is the thickness of the cuticle in the leaf, which minimizes how much of the active principle the plant absorbs.

According to a study [54], about 785,300 tons of products containing glyphosate were marketed worldwide in 2017. The results of histochemical tests were negative for detecting phenolic compounds and starches, and these results indicate that the species did not undergo oxidative stress [55]. No accumulation of starch grains was observed in *H. stigonocarpa* leaves, indicating that carbohydrate translocation was not impaired, as already observed in species exposed to various atmospheric pollutants [56].

Glyphosate is one of the most studied herbicides by the scientific community in recent years [57]. Glyphosate foliar phytotoxicity is related to absorption through the cuticle, in which case the removal of the wax layer due to herbicide application increases the chance of glyphosate absorption [58], which is why *H. stigonocarp* did not present visible or anatomical damage. Epicuticular wax is a very effective barrier against the foliar absorption of water-soluble herbicides, such as glyphosate [59]. For this reason, it may indicate that only sub-doses of glyphosate were absorbed by the plants, providing benefits to the studied plants.

Non-photochemical quenching was not affected by the glyphosate application, so the dissipation of non-radiative energy 24 hours and 60 days after the exposure of the seedlings to the herbicide was not affected. We know that non-photochemical quenching is involved in dissipating excess energy and regulating the photosystem II reaction center, which in turn is a photoprotection mechanism [60, 61]. The initial increase in the transpiration rate after 24 hours has already been pointed out as a defoliation compensation mechanism [62], which in turn could not be maintained during the 60 days as there was a progressive decrease, which may suggest that the plant's capacity to absorb enough water to replace that consumed in the transpiration process was not sufficient, as has been shown by other authors [62, 63].

Glyphosate acts by inhibiting the activity of the 5-enolpyruvylshikimate-3 phosphate synthase (EPSPS) enzyme, which catalyzes the condensation of shikimic acid and pyruvic acid, preventing the synthesis of three aromatic amino acids (e.g., phenylalanine, tyrosine and tryptophan), negatively influencing plant growth [15]. This happened in treated plants which had their growth affected depending on the glyphosate dose, as many herbicides were created with the objective of regulating growth [3].

Conclusion

The plants are tolerant to glyphosate with regard to visible and anatomical damage, and the lowest herbicide doses had little effect on the physiological analyses, which resulted in higher growth rates for the seedlings; however, the presence of the glyphosate in the highest doses negatively affected the foliar apparatus of plants, in turn affecting their growth.

Declarations

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Author contributions

GSF: conduct experiments and collected data, writing the draft manuscript and editing. LC: supervision, writing, review, and editing, project administration. AJ: supervision, review and editing. SCVF: supervision, conceptualization, analysed sample and data. STFF: formal analysis, visualization, review and editing. LLL: supervision and collected data. AMC: analysed sample and data. IOFS: analysed sample and data.

Data availability statement

Data may be made available by contacting the corresponding author.

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflicts of interest.

Ethics Statement

This study did not use any animal. The cultivars were obtained in accordance with Brazilian legislation, following the regulation of Law Nº 9,456, of April 25, 1997.

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Figures

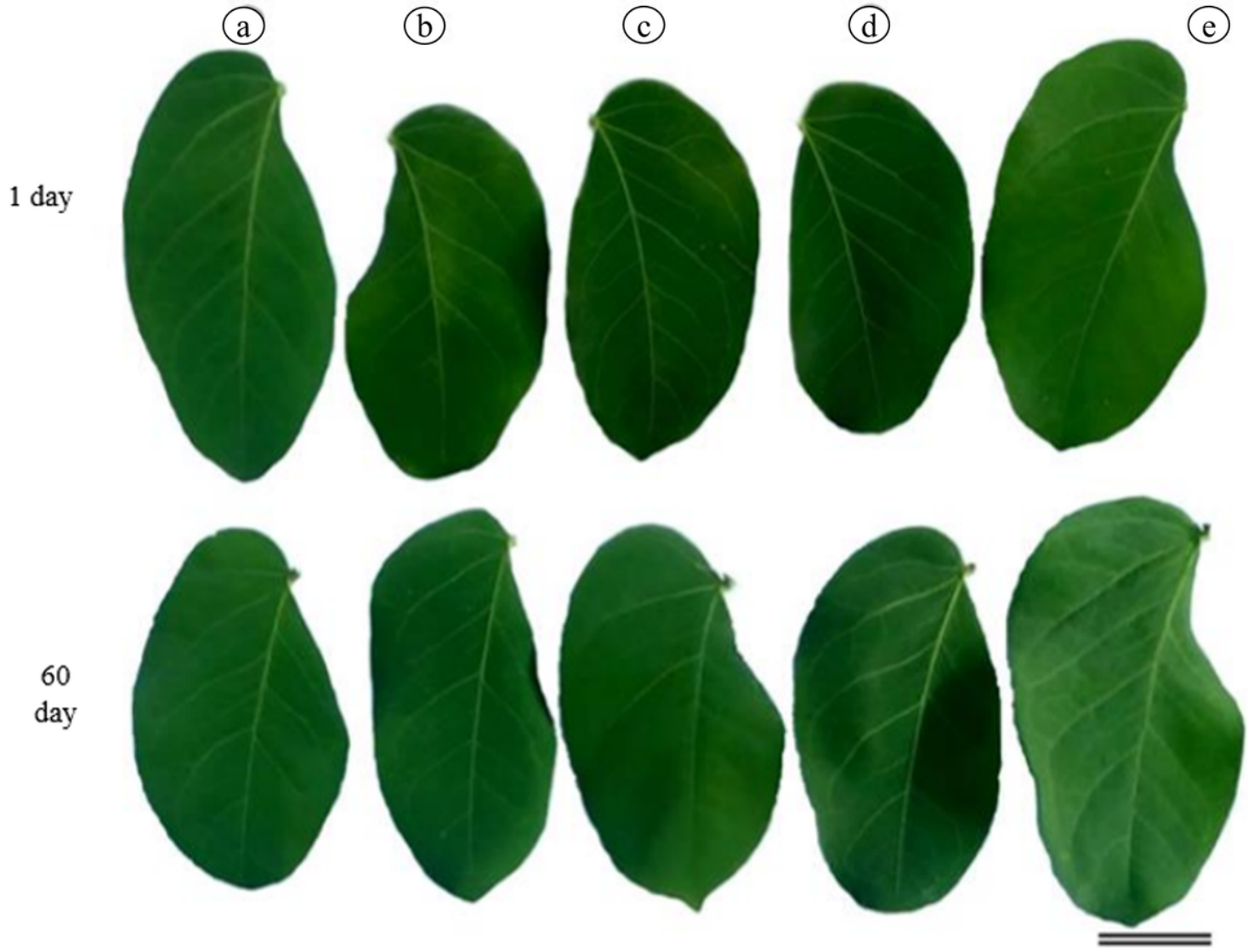

Figure 1

Leaves of *H. stigonocarp* Mart. ex Hayne. 24 hours and 60 days after treatment applications. (a) control, (b) 96 g ha^{-1}, (c) 240 g ha^{-1}, (d) 480 g ha^{-1}, (e) 960 g ha^{-1}. Scale bar 3 cm.

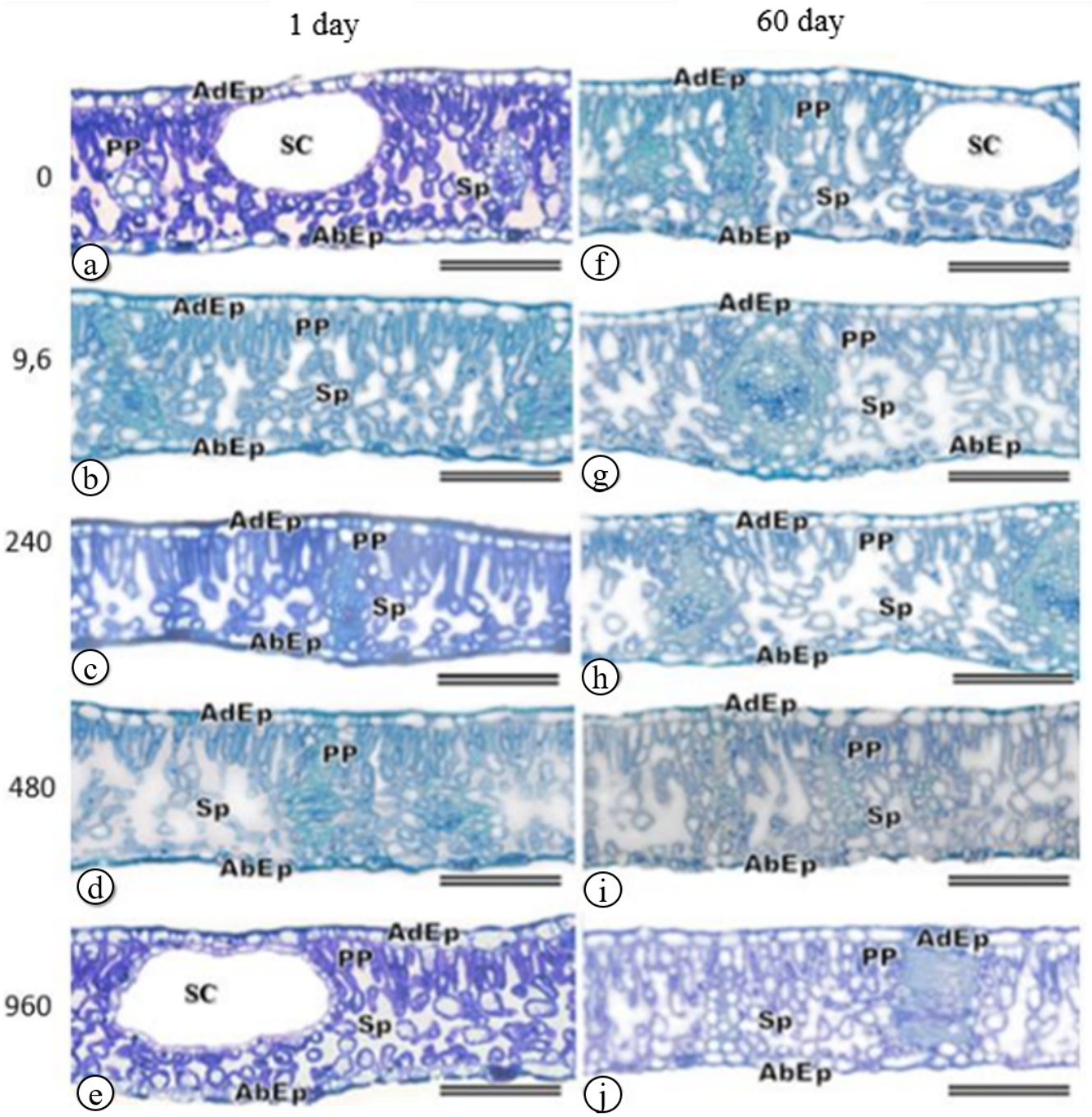

Figure 2

Leaf anatomical structures of *H. stigonocarpa* Mart. ex Hayne. 1 day and 60 days after treatment applications. (a) control, (b) 96 g ha^{-1}, (c) 240 g ha^{-1}, (d) 480 g ha^{-1}, (e) 960 g ha^{-1}, (f) control, (g) 96 g ha^{-1}, (h) 240 g ha^{-1}, (i) 480 g ha^{-1}, (j) 960 g ha^{-1}. (AdEp) adaxial epidermis. (AbEp) abaxial epidermis. (PP) palisade parenchyma. (SP) spongy parenchyma. (SC) secretory channel. (a-f) Scale bar 100 µm.

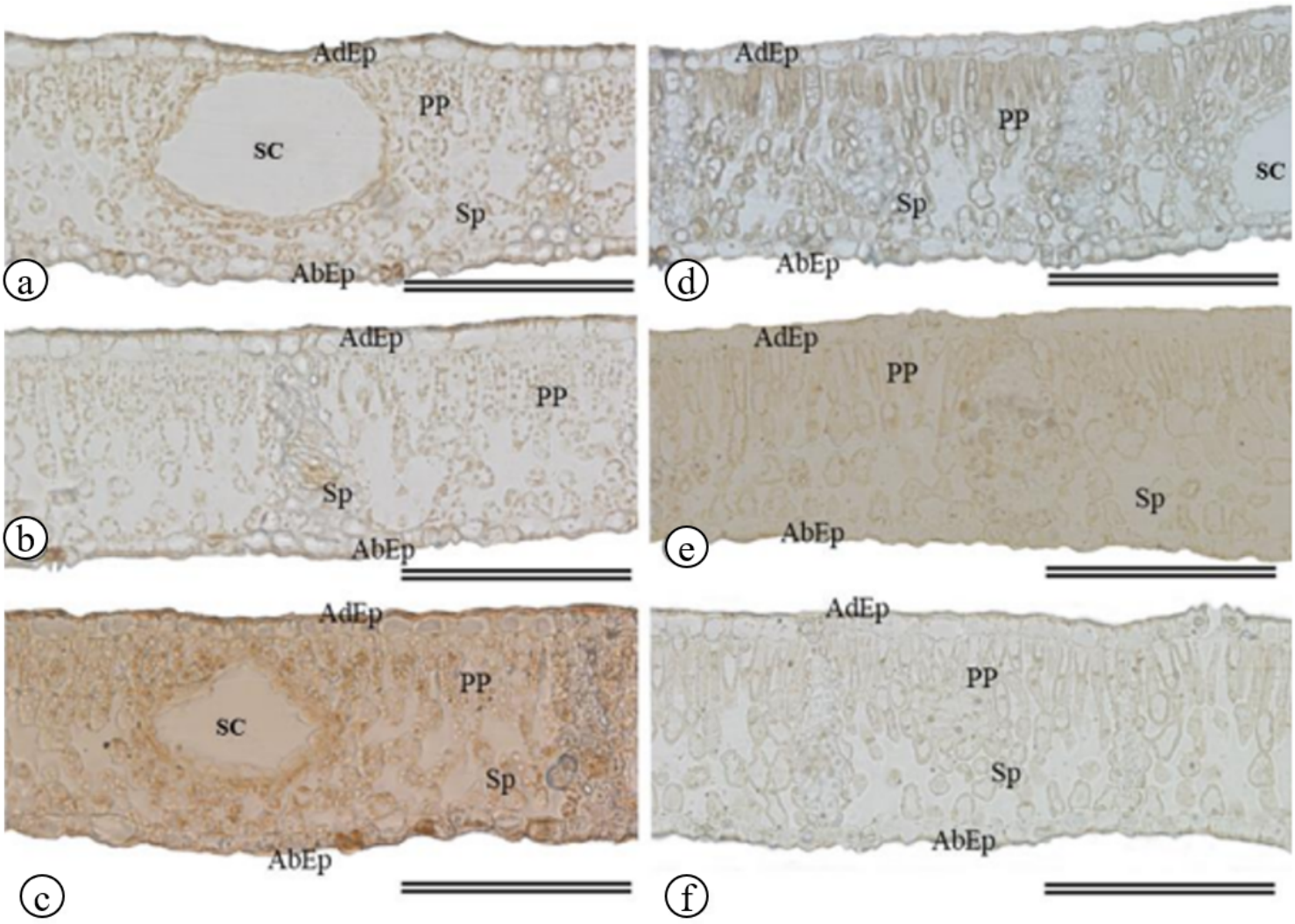

Figure 3

Starch grain accumulation in *H. stigonocarpa* Mart. ex Hayne. seedlings 60 days after glyphosate application of different increasing glyphosate doses. (a) control, (b) 96 g ha^{-1}, (c) 240 g ha^{-1}, (d) 480 g ha^{-1}, (e) 960 g ha^{-1}, (f) 960 g ha^{-1}. (AdEp) epidermis adaxial. (AbEp) abaxial epidermis. (PP) palisade parenchyma. (SP) spongy parenchyma. (a-f) Scale bar 100 µm.

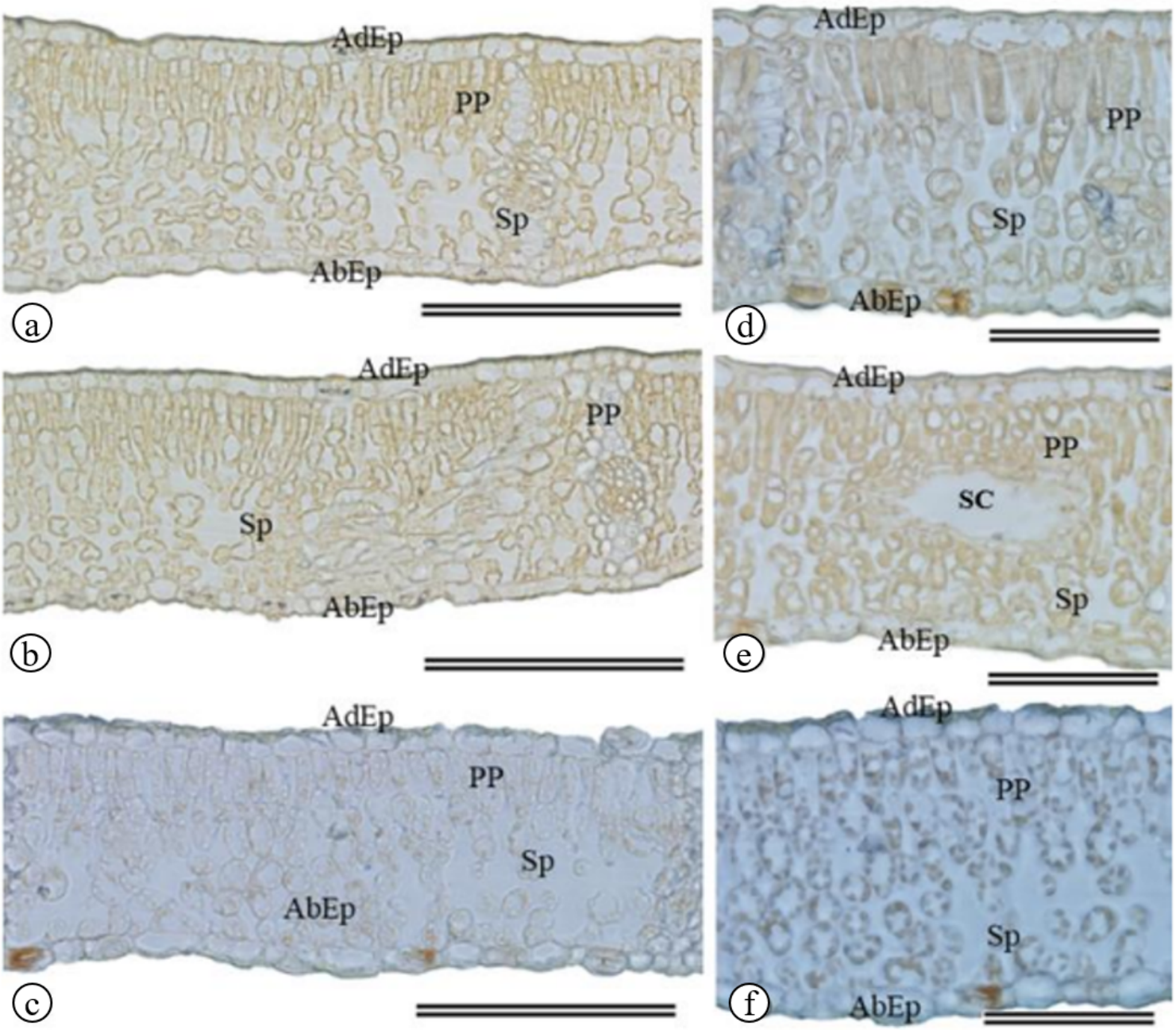

Figure 4

Phenolic compounds in *H. stigonocarpa* Mart. ex Hayne. seedlings 60 days after glyphosate application of different increasing glyphosate doses (a) control, (b) 96 g ha^{-1}, (c) 240 g ha^{-1}, (d) 480 g ha^{-1}, (e-f) 960 g ha^{-1}. (AdEp) adaxial epidermis. (AbEp) abaxial epidermis. (PP) palisade parenchyma. (SP) spongy parenchyma. (SC) secretory channel; (a-f) Scale bar 200 µm

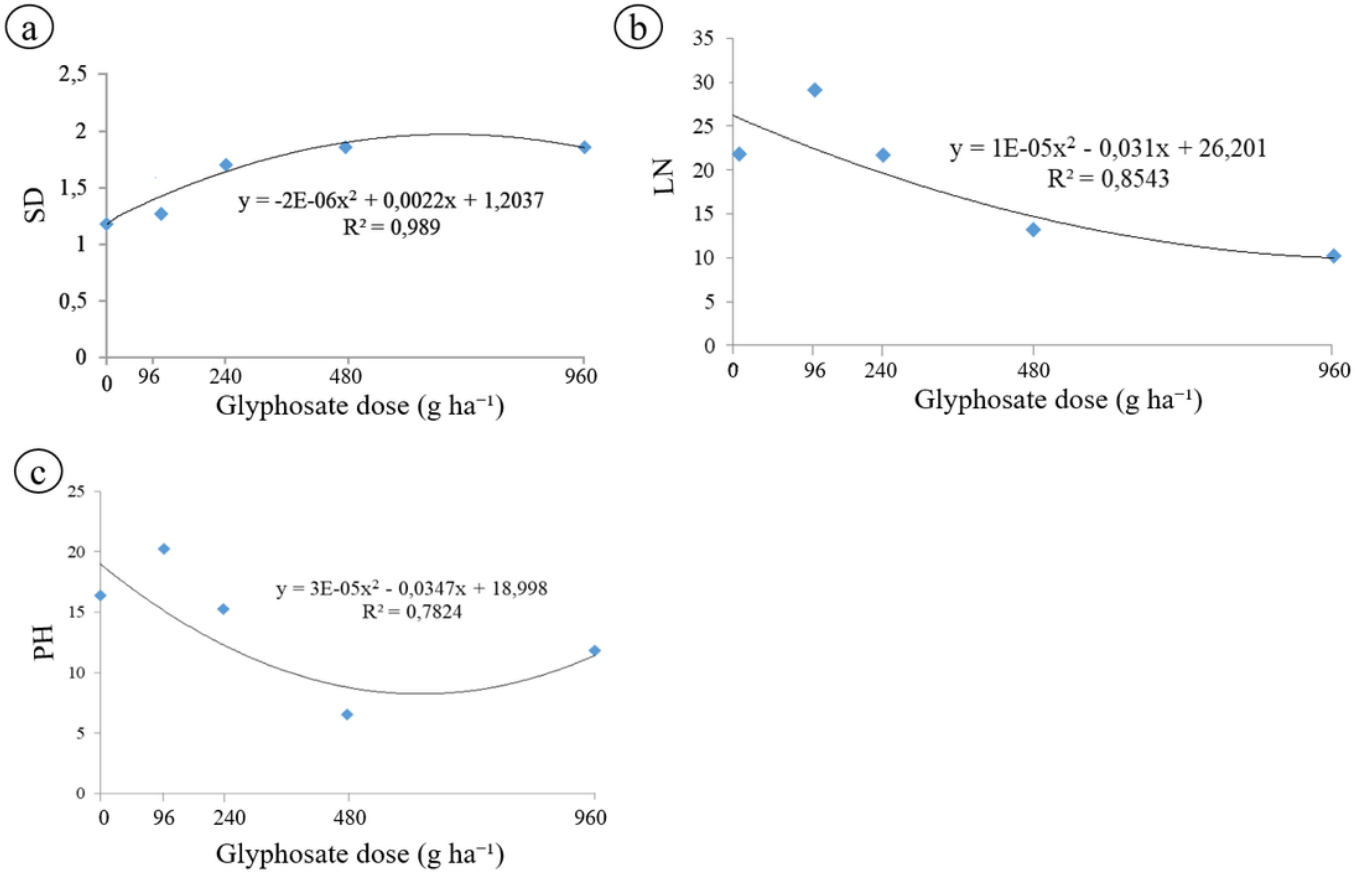

Figure 5

Plant height (PH) (a), stem diameter (SD) (b), and leaf number (LN) (c) of *H. stigonocarpa* Mart. ex Hayne. seedlings 60 days after glyphosate application.

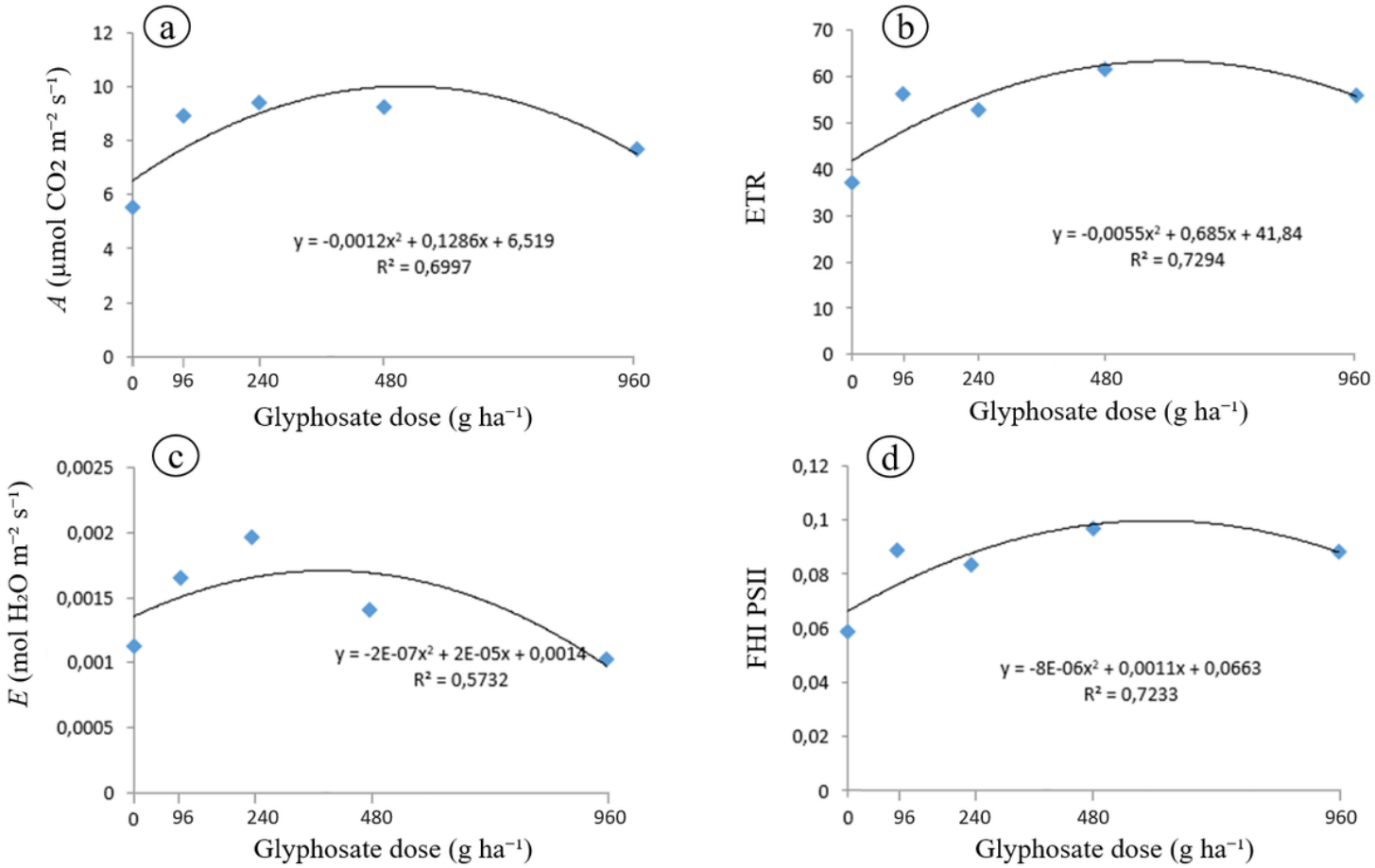

Figure 6

(a) Net photosynthetic rate, (b) Electron transport rate, (c) potential quantum yield of photosystem II, (d) effective quantum yield of photosystem II in *H. stigonocarpa* Mart. ex Hayne. seedlings 60 days after glyphosate application.

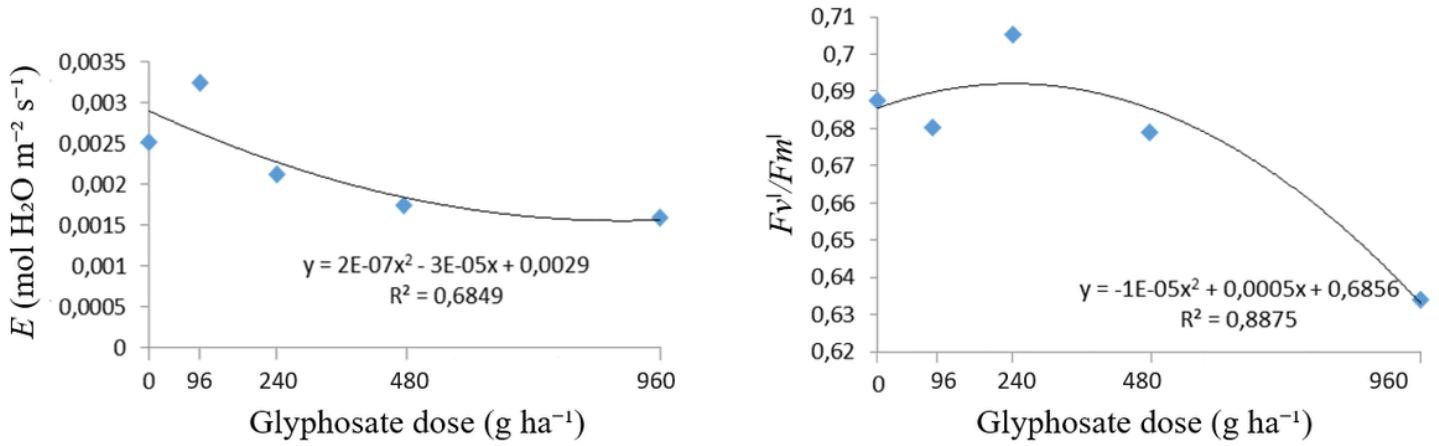

Figure 7

(a) Photosynthetic rate and (b) Transpiratory rate in *H. stigonocarpa* Mart. ex Hayne. seedlings 60 days after glyphosate application.