

PHYSIOLOGICAL AND MORPHOANATOMIC RESPONSES OF *Bowdichia virgilioides* KUNTH. (FABACEAE) TO GLYPHOSATE

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ABSTRACT –The expansion of agribusiness in Brazil is partly due to the expansion of arable areas in the Cerrado biome, which, consequently, leads to an increase in the use of herbicides, including glyphosate. Therefore, the objective of this study was to evaluate the physiological and morphoanatomic responses of *Bowdichia virgilioides* Kunth. (Fabaceae), when exposed to the herbicide glyphosate, under controlled greenhouse conditions. In addition, the study proposed to identify possible response patterns of the studied species and provide subsidies for the study of the species in areas affected by the drift of the herbicide. The experimental design was in randomized blocks, with seven doses of the herbicide: 25, 50, 100, 200, 400, 800, and 1200 g i.a ha⁻¹ besides the control (0), with four repetitions each. Gas exchange, chlorophyll a fluorescence emission, chloroplast pigment content, membrane permeability, and visual and anatomical features were analyzed. The results showed that glyphosate is phytotoxic to *B. virgilioides* because, even at low doses, this herbicide was able to affect all parameters analyzed. There was also confirmation of the presence of phenolic compounds, mainly in the palisade parenchyma. The metabolic alterations presented demonstrated the sensitivity of *B. virgilioides* to glyphosate. Thus, the observed responses can serve as a tool for diagnosing the effects of glyphosate exposure on this species, present in native areas affected by the drift of this herbicide. Its indiscriminate use poses a risk to Cerrado biodiversity.

Keywords: Herbicide; Phytotoxicity; Cerrado.

RESPOSTAS FISIOLÓGICAS E MORFOANATÔMICAS DE *Bowdichia virgilioides* KUNTH. (FABACEAE) AO GLIFOSATO

RESUMO – A expansão do agronegócio no Brasil, se deve em parte pela ampliação das áreas agricultáveis no Cerrado, o que, conseqüentemente, leva a um aumento do uso de herbicidas, entre eles o glifosato. Em vista disso, objetivou-se avaliar as respostas fisiológicas e morfoanatômicas de *Bowdichia virgilioides* Kunth. (Fabaceae), quando expostas ao herbicida glifosato, em condições controladas de casa de vegetação. Além disso, o estudo se propôs a identificar possíveis padrões de respostas da espécie estudada, com o propósito de fornecer subsídios para o estudo da espécie, em áreas atingidas por deriva deste herbicida. O delineamento experimental foi em blocos ao acaso, com sete doses do herbicida: 25, 50, 100, 200, 400, 800 e 1200 g i.a ha⁻¹, além do controle (0), com quatro repetições cada. Foram analisados: trocas gasosas, emissão de fluorescência da clorofila a, conteúdo de pigmentos cloroplastídicos, permeabilidade de membrana e características visuais e anatômicas. Os resultados mostraram que o glifosato é fitotóxico para *B. virgilioides*, pois, mesmo em doses baixas este herbicida foi capaz de afetar todos os parâmetros analisados. Houve também a confirmação da presença de compostos fenólicos, principalmente no parênquima paliçádico. As alterações metabólicas apresentadas, comprovam a sensibilidade de *B. virgilioides* ao glifosato. Dessa maneira, as respostas



observadas, podem servir como ferramenta de diagnóstico dos efeitos da exposição de glifosato sobre essa espécie, presente em áreas nativas atingidas pela deriva deste herbicida. Seu uso indiscriminado é um risco à biodiversidade do Cerrado.

Palavras-Chave: Herbicida; Fitotoxicidade; Cerrado.

1. INTRODUCTION

Brazilian agriculture has grown at an accelerated pace, influenced by the exploration of new arable areas, associated with the modernization of machinery and implements, and the strengthening of the input sector (Belchior et al., 2014). In the last four decades, the expansion of agriculture in the Cerrado was the one that most contributed to the increase of the sector in the country, standing out in the production of soybeans, corn, cotton, sugar cane, sorghum and rice (Santana et al., 2020). However, even though it is of great importance for the Brazilian economy, agribusiness has caused several impacts to natural resources, mainly due to the exacerbated use of pesticides, which directly affect the soil, water resources and biodiversity (Gomes, 2019). In this market, herbicides, insecticides and fungicides account for 95% of the world consumption of pesticides (Pelaez et al., 2010).

Among the most widely used herbicides worldwide, glyphosate (N-phosphonomethyl glycine) stands out (Benbrook, 2016). This herbicide acts directly on the enzyme 5-enolpyruvyl-shikimate-3-phosphatesynthase (EPSPS), inhibiting it, affecting the metabolic route of shikimic acid, which reduces the synthesis of essential aromatic amino acids, such as phenylalanine, tyrosine and tryptophan (Kirkwood and McKay, 1994; Freitas-Silva et al., 2020; Santos et al., 2020). One of the most expressive characteristics of glyphosate is its rapid translocation, resulting in total destruction of plants in a short period, also having action on seeds in the soil, thus expressing its high degree of toxicity (Yamada and Castro, 2007). Thus, it is necessary to investigate the phytotoxic effects caused by glyphosate drift on native vegetation (Freitas-Silva et al., 2020).

Some research has evaluated the damage caused by glyphosate drift on native species in controlled environments, Silva et al. (2016), demonstrated that this herbicide affected all photosynthetic variables of the species *Caryocar brasiliense* Camb., as well as Batista et al. (2018), in *Pouteriatorta*. Santos et al. (2020), found that in the species *Cenostigma*

macrophyllum Tul., glyphosate caused photosynthetic and anatomical damage, the same was described by Freitas-Silva et al. (2020), in the species *Handroanthus chrysotrichus* and *Garcinia gardneriana*, and Cruz et al. (2021), in *Eugenia uniflora* L. However, there is still a lack of studies on the potential damage to forest species caused by the application of herbicides (Brancaion et al., 2009). Therefore, prior knowledge of the morphoanatomical and physiological responses of native plants exposed to herbicides, acquired in controlled environments is essential, as they will serve as a tool to support the monitoring of the effects of glyphosate drift in natural vegetation.

In view of the suppression of the natural vegetation of the Cerrado caused by the expansion of agribusiness, the choice of *Bowdichia virgilioides* Kunth. for this study occurred because it is a native species, of wide distribution, found in the Cerrado, Caatinga, Pantanal, and Atlantic Forest, and because it is classified as a pioneer plant, tolerant to dry and poor soils, and with high potential for use in the recovery of degraded areas and reforestation, besides being highly valued economically for its ornamental, medicinal, and timber potential. The species belongs to the Fabaceae family, subfamily Papilionoideae, popularly known as sucupira-preta or paricarana, its leaves are compound, imparipinnate and alternate spiral (Lorenzi, 2008; Coêlho et al., 2019).

Therefore, this study aimed to evaluate the physiological and morphoanatomical responses of *Bowdichia virgilioides* Kunth (Fabaceae), when exposed to glyphosate herbicide, under controlled conditions in a greenhouse, and to identify possible patterns of responses of this exposure by the studied species, in order to provide subsidies for the study of the species in areas affected by drift of this herbicide.

2. MATERIAL AND METHODS

2.1. Cultivation conditions and experimental design

The experiment was carried out in a greenhouse, with controlled temperature and humidity. The

minimum temperature recorded was 23.9°C and the maximum 29°C. The relative humidity of the air varied between 60.2% and 80.9%. Before planting, the seeds of the species *B. virgilioides* were immersed in concentrated sulfuric acid (98%) for 8 minutes, then washed in running water for 10 minutes to break dormancy, then placed to germinate in plastic trays containing sand. After 45 days, the seedlings were transplanted in polyethylene pots containing 3 kg of substrate, which consisted of a mixture of two parts of soil, type dystrophic red latosol and one of fine sand. In each pot a plant was grown, which every twenty days received a nutrient solution from Hoagland and Arnon (1950), (half strength).

Treatments were carried out 6 months after transplantation. At the time of glyphosate application, the plants were on average 20 cm tall, with two to four leaves on the main branch, containing three to five leaflets. The design was in randomized blocks, with seven doses of the herbicide, being: 25, 50, 100, 200, 400, 800 and 1200 g i.a ha⁻¹ (grams of active ingredient per hectare) and the control (0), with four replicates for each. For application, a backpack sprayer (constant pressure maintained by CO₂) was used, equipped with a four-point bar (XRTeeJet®) of the 110.02 VP series, calibrated for the application of 180L ha⁻¹ of syrup. The spraying was done only once, in the four repetitions of each dose, at the same time.

2.2. Physiological assessments

For gas exchanges, six measurements were made (24, 48, 216, 244, 264 and 336 hours, after application), in fully expanded leaflets, in the median portion of the plant. In addition to gas exchange, the same area was used to assess chlorophyll a fluorescence. The photosynthetic (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$), transpiratory (E , $\text{mmol m}^{-2} \text{s}^{-1}$), stomatal conductance (g_s , $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$) and internal to external CO₂ concentration (C_i/C_a) ratios were measured using a Li-COR portable photosynthesis meter (LI 6400 XT, LiCor, USA). For chlorophyll a fluorescence, ten measurements were performed (24, 48, 72, 120, 144, 168, 216, 244, 264 and 336 hours after glyphosate application), assessed using a portable modulated fluorometer (MINI-PAM, Walz, GER), with tweezers, model 2030-B (Bilger et al., 1995; Rascher et al., 2000). The potential quantum yield of photosystem II (Van Kooten and Snel, 1990) was calculated after 30 minutes of adaptation to the dark.

The evaluations of the content of chloroplast pigments and membrane permeability occurred 336 hours after the application of glyphosate. The pigments were extracted according to the methodology described by Wellburn (1994), and Moura et al. (2018). Three leaf discs were collected from each repetition, avoiding the midrib. These were incubated in glass flasks wrapped in aluminum foil, containing 5ml of DMSO saturated with CaCO₃. Then they were placed in a water bath at a temperature of 65°C for 24 hours. The absorbance was determined on the UV – VIS spectrophotometer, Evolutin 60S (Thermo Fischer Scientific, USA). The wavelengths and the equations for calculating the concentrations of chlorophyll *a*, *b* and chlorophyll *a/b* ratio, were based on the work of Wellburn (1994). The permeability of membranes was determined using the electrolyte release rate. Fifteen 0.2cm² leaf discs were collected per repetition, immersed in 30ml of deionized water in glass flasks, left to stand for 24 hours. After this period, the free electrical conductivity and the total conductivity were measured, according to the methodology described by Vasquez-Tello et al. (1990) and Pimentel et al. (2002).

2.3. Visual assessment of leaf symptoms

In order to assess visual phytotoxicity, six observations were made (24, 72, 120, 168, 240 and 336 hours, after the application of glyphosate), by adjusting the methodology described by Novak et al. (2003). The number of leaves with visible symptoms was verified according to the percentage of affected area considering the classes: 5% (N1), > 5% to 25% (N2), > 25% to 50% (N3), > 50% at 75% (N4) and >75% to 100% (N5). In addition, the number of leaves without symptoms (N0) per plant was counted. The number of leaves in each class (N0 to N5) was used to calculate the leaf injury index (LII) per plant using the formula adapted by El-Khatib (2003):

$$\text{LII (\%)} = \frac{(N1 \times 1) (N2 \times 2) (N3 \times 3) (N4 \times 4) (N5 \times 5) \times 100}{(N0 + N1 + N2 + N3 + N4 + N5) \times 5}$$

The severity (SI) of visible symptoms per plant was also calculated, dividing the number of leaves with symptoms by the total number of leaves of the plant, which was also expressed as a percentage.

$$\text{SI (\%)} = \frac{\text{Number of leaves with symptoms} \times 100}{\text{Total sheets}}$$

2.4. Anatomical analyzes

For light microscopy, four samples of each treatment were collected, 336 hours after the application

of the herbicide. The fully expanded leaflets were removed, and cuts were made in the median region, which were fixed in a 1% Glutaraldehyde solution, in 0.2M phosphate buffer, pH 7.2 (Karnovsky, 1965), for 24 hours, and stored in 70% alcohol. They were then dehydrated, at a time interval of 1 hour for each exchange, in ethyl and butyl series (80, 90 and 100%, ethyl butyl (3:1), ethyl butyl (1:1), ethyl butyl (1:3) and pure butyl, remaining over night in butyl alcohol + paraffin (1:1), then they were submitted to two paraffin changes for 1 hour each. After infiltration, the samples were packed (paraffin + 8% beeswax), following the orientation of the cutting plane (transverse), with four blocks for each treatment, one for each repetition, totaling 32 blocks.

For anatomical characterization, transverse sections were made in a semi-motorized rotating microtome (RM2245, Leica, GER), 10 μ m thick and adhered to the slide with Haupt adhesive (Haupt, 1930). Three slides were made for each block. The sections were deparaffinized in xylolic series, hydrated in ethyl series and stained in 1% safranin and blue for 20 min (Gerlach, 1984). Then, the slides were washed in distilled water and dehydrated in an ethylic series (30%, 50%, 70%, 85%, 95% and 100%), then submitted in xylol series and mounted between slide and coverslip in balsam from Canada. Three sections were randomly selected on each slide. Images were captured by a Leica optical microscope (DM 500, Leica, GER) with a Leica camera (ICC50 HD, Leica, GER) attached. For micromorphometry, images obtained under a 40x objective, from the images, five measurements were made in each section, using the ANATI QUANTI software, version 2.0 for Windows® (Aguiar et al., 2007).

For the histochemical tests (phenolic compounds and tannins), sections obtained on a tabletop microtome (ROBHE/LPC, BRA), were submitted to ferric chloride and hydrochloric vanillin, at doses of (200 g i.a ha⁻¹) and (800 g i.a ha⁻¹). Cuts without any reagent were called white.

2.5. Statistical analysis

The data obtained for the morphometric analyzes were submitted to analysis of variance (ANOVA), and the means were compared using the Tukey test, at a 5% significance level. Statistical analyzes were performed using the Statistica 5.0 program. The data

from the physiological and phytotoxicity analyzes were subjected to analysis of variance (ANOVA) and the regression models were adjusted. Graphs and statistical analyzes were performed using SigmaPlot 10.0 software.

3. RESULTS

3.1. Physiological analyzes

The gas exchange parameters of *B. virgilioides* were affected by glyphosate (Figure 1). Decrease in the values of photosynthetic rate (*A*) (Figure 1 and Table 1), stomatal conductance (*g_s*) (Figure 1B) and transpiratory rate (*E*) (Figure 1C) and increase in the relationship between internal and external CO₂ concentration (*C_i/C_a*) (Figure 1D), were verified according to the increase in applied doses. Comparing the highest dose 1200 g i.a ha⁻¹, with the control, *A* showed a reduction, on average, of more than 50% in all evaluations, and in the evaluation of 216 hours after the application, this value reached 99.9%. For *g_s* these decreases were, on average, 36%, presenting 58.8% in 216 hours. The reduction in *E* followed the same pattern of *g_s*, about 30% on average, in all evaluations being 216 hours, with 53% less than the control. In contrast, the *C_i/C_a* ratio showed more expressive increases in the 216 hours analysis, after application, increasing by 92.2%, with a dose of 1200 g i.a ha⁻¹, compared to the control.

Changes in the fluorescence parameters of chlorophyll *a* occurred in all doses according to the increase in its concentrations (Figure 1 and Table 1). The values of the potential quantum yield of photosystem II (*F_v / F_m*) (Figure 1E), were significantly reduced until the analysis of 240 hours after application. The same behavior was observed for the effective quantum yield of photosystem II ($\Delta F / F_m'$) (Figure 1F) and apparent electron transport rate (ETR) (Figure 1G). There was an increase in the non-photochemical extinction coefficient (NPQ) (Figure 1H), in all evaluations, but the values were only significant in the analyzes of 24, 120 and 336 hours after application, and in the last evaluation the value with 1200 g i.a ha⁻¹ dose was 36.5% higher than the control.

The results obtained for the content of chloroplast pigments (Figure 2), showed significant differences in relation to the doses applied for chlorophyll *a*

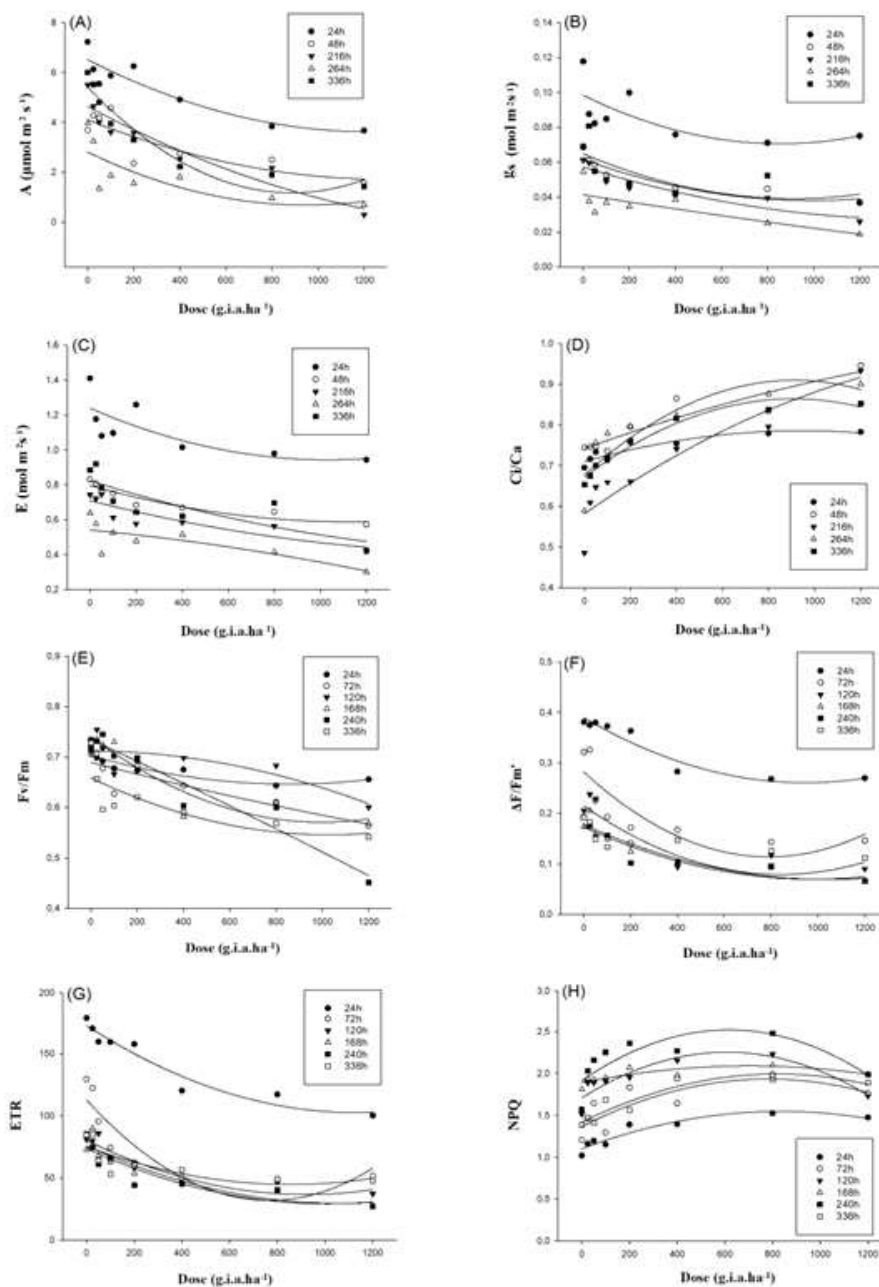


Figure 1 – A-D: Effects of glyphosate on *Bowdichia virgilioides* gas exchange parameters. A: photosynthetic rate (A); B: transpiration rate (E); C: stomatal conductance (g); D: C/C_a ratio. E-H: Effects of glyphosate on chlorophyll *a* fluorescence parameters from *Bowdichia virgilioides*. E: potential quantum efficiency of photosystem II (F_v / F_m); F: effective quantum yield of photosystem II (ΔF/F_m); G: electron transport rate (ETR); H: non-photochemical dissipation coefficient (NPQ). For all parameters, the adjusted regression model was quadratic polynomial. Significance * p < 0.05%.

Figura 1 – A-D: Efeitos do glifosato nos parâmetros de trocas gasosas de *Bowdichia virgilioides*. A: taxa fotossintética (A); B: taxa transpiratória (E); C: condutância estomática (g); D: razão C/C_a. E-H: Efeitos do glifosato nos parâmetros de fluorescência da clorofila *a* de *Bowdichia virgilioides*. E: rendimento quântico potencial do fotossistema II (F_v/F_m); F: rendimento quântico efetivo do fotossistema II (ΔF/F_m); G: taxa de transporte de elétrons (ETR); H: coeficiente de dissipação não fotoquímica (NPQ). Em todos os parâmetros o modelo de regressão ajustado foi polinomial quadrático. Significância * p < 0,05%.

Table 1 – Adjusted regression equations for gas exchange and chlorophyll *a* fluorescence in *B. virgilioides* plants subjected to different concentrations of glyphosate. They refer to Figure 1.

Tabela 1 – Equações de regressão ajustadas para trocas gasosas e fluorescência da clorofila *a*, em plantas de *B. virgilioides* submetidas a diferentes concentrações de glifosato. Referem-se à Figura 1.

Features	Unit	Adjusted equations	R ²
<i>A</i>	24 hours after application	$y = 6,5181 - 0,0047x + 2E-06x^2$	0,85*
<i>A</i>	48 hours after application	$y = 4,1068 - 0,0037x + 1E-06x^2$	0,72*
<i>A</i>	216 hours after application	$y = 4,6425 - 0,0049x + 1E-06x^2$	0,90*
<i>A</i>	264 hours after application	$y = 2,8092 - 0,0045x + 2E-06x^2$	0,56 ^{ns}
<i>A</i>	336 hours after application	$y = 5,4184 - 0,0096x + 5E-06x^2$	0,92*
<i>g_s</i>	24 hours after application	$y = 0,0985 - 7E-05x + 4E-08x^2$	0,49 ^{ns}
<i>g_s</i>	48 hours after application	$y = 0,00574 - 4E-05x + 1E-08x^2$	0,82*
<i>g_s</i>	216 hours after application	$y = 0,0493 - 2E-05x + 2E-10x^2$	0,88*
<i>g_s</i>	264 hours after application	$y = 0,0414 + 2E-05x - 2E-09x^2$	0,61 ^{ns}
<i>g_s</i>	336 hours after application	$y = 0,065 - 6E-05x + 3E-08x^2$	0,50 ^{ns}
<i>E</i>	24 hours after application	$y = 1,2393 - 0,0006x + 3E-07x^2$	0,57 ^{ns}
<i>E</i>	48 hours after application	$y = 0,796 - 0,0004x + 2E-07x^2$	0,89*
<i>E</i>	216 hours after application	$y = 0,7094 - 0,0003x + 9E-08x^2$	0,80*
<i>E</i>	264 hours after application	$y = 0,5419 + 0,0001x - 6E-08x^2$	0,62 ^{ns}
<i>E</i>	336 hours after application	$y = 0,8238 - 0,0004x + 1E-07x^2$	0,68 ^{ns}
Ci/Ca	24 hours after application	$y = 0,7102 - 0,0002x + 8E-08x^2$	0,85*
Ci/Ca	48 hours after application	$y = 0,7418 + 0,0002x + 4E-08x^2$	0,86*
Ci/Ca	216 hours after application	$y = 0,6774 + 0,0005x - 3E-07x^2$	0,88*
Ci/Ca	264 hours after application	$y = 0,6774 + 0,0005x - 3E-07x^2$	0,79*
Ci/Ca	336 hours after application	$y = 0,672 + 0,0004x - 2E-07x^2$	0,96*
Fv/Fm	24 hours after application	$y = 0,7028 - 0,0001x + 8E-08x^2$	0,84*
Fv/Fm	72 hours after application	$y = 0,6893 - 0,0001x + 1E-08x^2$	0,80*
Fv/Fm	120 hours after application	$y = 0,7125 - 3E-06x - 7E-08x^2$	0,70*
Fv/Fm	168 hours after application	$y = 0,7398 - 0,0003x + 2E-07x^2$	0,88*
Fv/Fm	240 hours after application	$y = 0,7343 - 0,0002x - 1E-08x^2$	0,94*
Fv/Fm	336 hours after application	$y = 0,6586 - 0,0002x + 1E-07x^2$	0,61 ^{ns}
ΔF/Fm'	24 hours after application	$y = 0,3897 - 0,0003x + 1E-07x^2$	0,93*
ΔF/Fm'	72 hours after application	$y = 0,282 - 0,0004x + 3E-07x^2$	0,72*
ΔF/Fm'	120 hours after application	$y = 0,215 - 0,0003x + 2E-07x^2$	0,77*
ΔF/Fm'	168 hours after application	$y = 0,1728 - 0,0002x + 1E-07x^2$	0,84*
ΔF/Fm'	240 hours after application	$y = 0,1728 - 0,0002x + 1E-07x^2$	0,84*
ΔF/Fm'	336 hours after application	$y = 0,1693 - 1E-04x - 5E-08x^2$	0,62 ^{ns}
ETR	24 hours after application	$y = 72,63 - 0,1241x + 5E-05x^2$	0,94*
ETR	72 hours after application	$y = 112,83 - 0,2112x + 0,0001x^2$	0,82*
ETR	120 hours after application	$y = 80,338 - 0,0952x + 5E-05x^2$	0,86*
ETR	168 hours after application	$y = 75,759 - 0,0871x + 4E-05x^2$	0,85*
ETR	240 hours after application	$y = 73,547 - 0,088x + 4E-05x^2$	0,81*
ETR	336 hours after application	$y = 75,32 - 0,0717x - 4E-05x^2$	0,63 ^{ns}
NPQ	24 hours after application	$y = 1,1007 + 0,0011x - 6E-07x^2$	0,90*
NPQ	72 hours after application	$y = 1,3663 + 0,0015x - 9E-07x^2$	0,62 ^{ns}
NPQ	120 hours after application	$y = 1,7087 + 0,0018x - 1E-06x^2$	0,80*
NPQ	168 hours after application	$y = 1,8894 + 0,0006x - 4E-07x^2$	0,64 ^{ns}
NPQ	240 hours after application	$y = 1,914 + 0,0002x - 2E-06x^2$	0,61 ^{ns}
NPQ	336 hours after application	$y = 1,4173 - 0,0014x + 9E-07x^2$	0,87*

(*), (ns) Significant and non-significant respectively, $p < 5\%$

(*), (ns) Significativo e não significativo respectivamente, $p < 5\%$

(Figure 2A), *b* (Figure 2B) and chlorophyll *a/b* ratio (Figure 2C). These changes were more evident from the lowest dose 25 g i.a ha⁻¹, for chlorophyll *a* and chlorophyll *b*, when compared to the control. For the chlorophyll *a/b* ratio, the decreases also occurred at

the lowest dose, but at the dose of 200 g i.a ha⁻¹, there was an increase in relation to all other doses. When compared to the control, the last dose 1200 g i.a ha⁻¹, there was a reduction of 36.5% and 21.7% in the levels of chlorophyll *a*, in chlorophyll *b*, respectively.

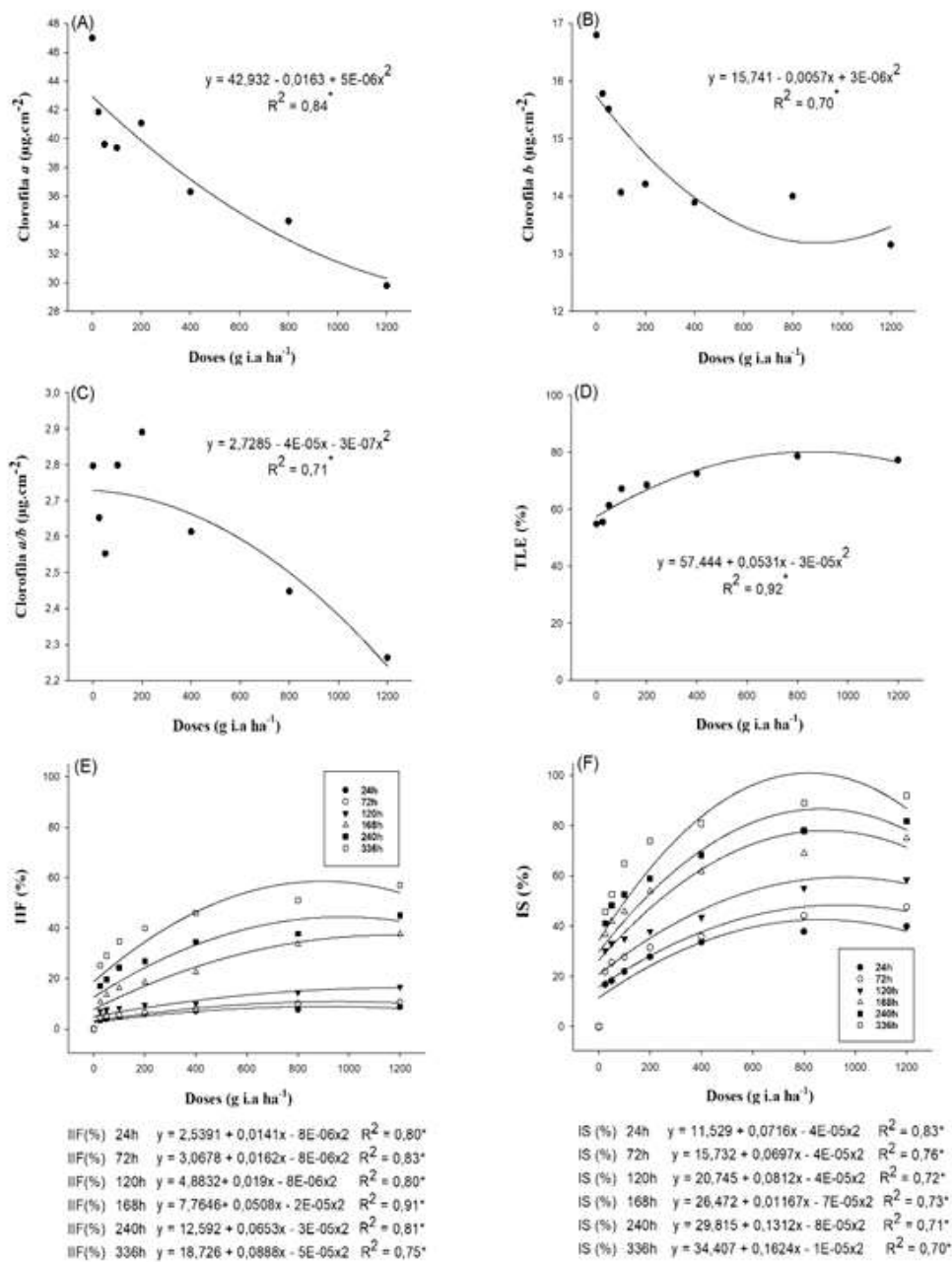


Figure 2 – A-C: Chloroplast pigment content of *Bowdichia virgilioides* plants subjected to different concentrations of glyphosate. A: chlorophyll a; B: chlorophyll b; C: chlorophyll a/b ratio; D: Electrolyte release rate in leaf tissues; E: Leaf injury index (IIF) and F: severity index (IS) after 24, 72, 120, 168, 240 and 336 hours after glyphosate application. Below the graphs are the regression equations adjusted for the Leaf Injury Index (LII) and Severity Index (SI). The adjusted regression model was quadratic polynomial. Significance: * $p < 0.05\%$.

Figura 2 – A-C: Conteúdo de pigmentos cloroplásticos de plantas de *Bowdichia virgilioides* submetida a diferentes concentrações de glifosato. A: clorofila a; B: clorofila b; C: razão clorofila a/b; D: Taxa de liberação de eletrólitos em tecidos foliares; E: Índice de injuria foliar (IIF) e F: índice de severidade (IS) após 24, 72, 120, 168, 240 e 336h da aplicação de glifosato. Abaixo dos gráficos encontram-se as equações de regressão ajustadas para Índice de injuria foliar (IIF) e índice de severidade (IS). O modelo de regressão ajustado foi polinomial quadrático. Significância: * $p < 0,05\%$.

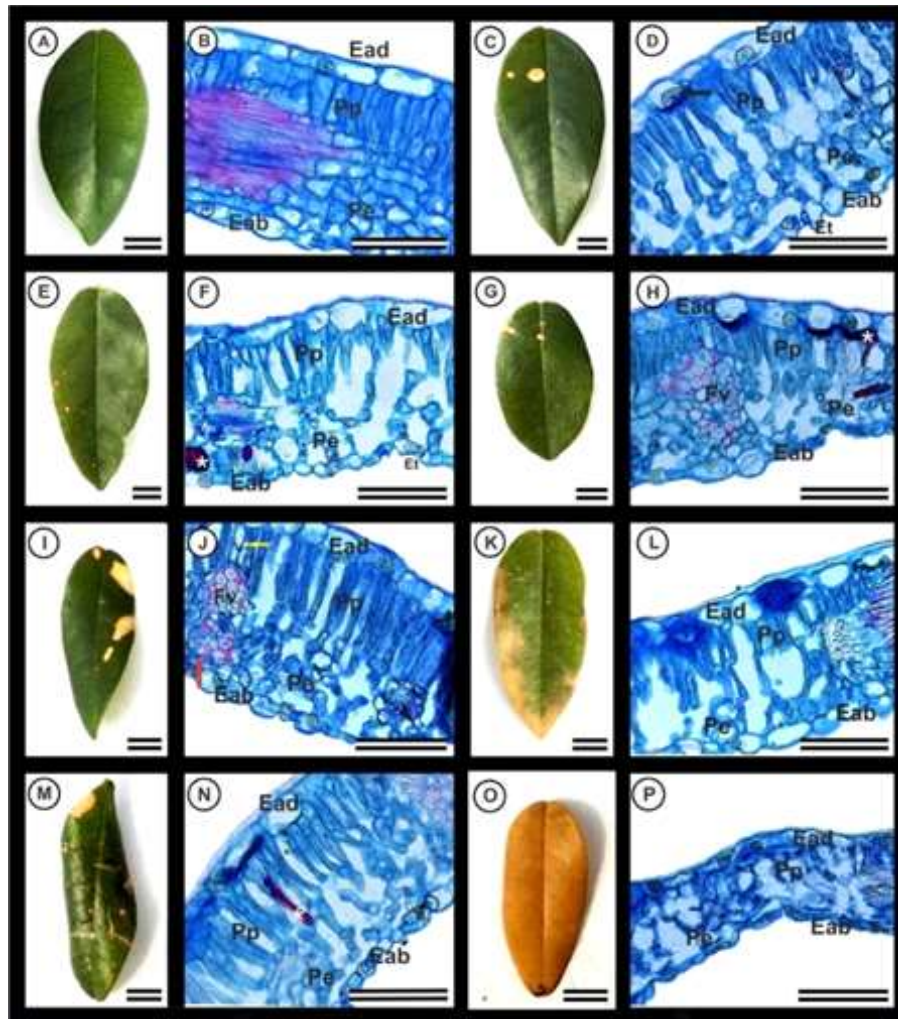


Figure 3 – Leaflet structure of *Bowdichia virgilioides* Khunt. Control (A and B); and subjected to the action of glyphosate: 25 g i.a ha⁻¹ (C and D); 50 g i.a ha⁻¹ (E and F); 100 g i.a ha⁻¹ (G and H); 200 g i.a ha⁻¹ (I and J); 400 g i.a ha⁻¹ (K and L); 800 g i.a ha⁻¹ (M and N); 1200 g i.a ha⁻¹ (O and P). Ead: adaxial epidermis; Eab: abaxial epidermis; Pp: palisade parenchyma; Pe: Spongy Parenchyma; Et: Stomata; Fv: Vascular bundle; Xi: Xylem; Fl: Phloem; Ba: Sheath; Tc: Trichoma; Prismatic crystals (→ red); (A, C, E, G, I, K, M and O) Bar = 1cm; (B, D, F, H, J, L, N, P) Bar = 50 μm.

Figura 3 – Estrutura do folíolo de *Bowdichia virgilioides* Khunt. Controle (A e B); e submetida à ação do glifosato: 25 g i.a ha⁻¹ (C e D); 50 g i.a ha⁻¹ (E e F); 100 g i.a ha⁻¹ (G e H); 200 g i.a ha⁻¹ (I e J); 400 g i.a ha⁻¹ (K e L); 800 g i.a ha⁻¹ (M e N); 1200 g i.a ha⁻¹ (O e P). Ead: Epiderme adaxial; Eab: epiderme abaxial; Pp: parênquima paliçádico; Pe: Parênquima esponjoso; Et: Estômato; Fv: Feixe vascular; Xi: Xilema; Fl: Floema; Ba: Bainha; Tc: Tricoma; Cristais prismáticos (→ vermelha); (A, C, E, G, I, K, M e O) Barra= 1cm; (B, D, F, H, J, L, N, P) Barra= 50 μm.

Regarding membrane permeability (Figure 2D), there was an increase in the rate of release of electrolytes from the dose of 50 g i.a ha⁻¹, with an increase of 11.9%, in relation to the control. When compared to the higher dose, with the control, this increase was 40.9%.

3.2. Visual assessment of leaf symptoms

There was a significant increase in LII and SI (Figure 2E and Figure 2F), observed from the lowest

dose to the highest dose and in the first evaluation, these damages increased as a result of the time after application. The most evident symptoms were chlorosis, leaf necrosis and, in the highest doses, the curling of some leaves. These effects were enhanced in the evaluation 168 hours after application, when the values for LII were increased by 10.8% for the lowest applied dose (25 g i.a ha⁻¹) and by 37.7% with the highest dose (1200 g i.a ha⁻¹), for SI the values were 36% and 75% respectively. In the last evaluation,

Table 2 – Leaf blade morphometry of *Bowdichia virgilioides* Kunth., after exposure to different doses of Glyphosate.
Tabela 2 – Morfometria da lâmina foliar de *Bowdichia virgilioides* Kunth., após exposição a diferentes doses de Glifosato.

Glyphosate	ADE (μm) [*]	PP (μm) [*]	SP (μm) [*]	ABE (μm) ^{ns}	LT (μm) ^{ns}
0	27,96 a	67,69 a	67,09 a	17,86 a	171,84 a
25	24,22ab	57,80ab	63,69ab	15,64 a	169,60 a
50	25,90ab	57,74 ab	57,63ab	14,72 a	165,28 a
100	24,69ab	56,56ab	57,63ab	15,22 a	186,82 a
200	24,43 ab	58,19 ab	61,72ab	16,63 a	179,38 a
400	24,75ab	52,61 ab	53,41ab	15,73 a	183,66 a
800	24,19 ab	55,53 ab	53,03ab	15,30 a	180,05 a
1200	19,63 b	43,73 b	42,40 b	14,66 a	144,82 a
CV%	11,8	14,28	13,06	15,01	11,94

(^{*}), (^{ns}) Significant and non-significant, respectively, at 5% probability by the F test. Means followed by the same letter in the column do not differ from each other at 5% probability by Tukey's test. ADE: adaxial epidermis; PP: Palisade parenchyma; SP: Spongy Parenchyma; ABE: abaxial epidermis; LT: Leaflet thickness.

(^{*}), (^{ns}) Significativo e não significativo respectivamente, a 5% de probabilidade pelo teste F. Médias seguidas pela mesma letra na coluna não diferem entre si a 5% de probabilidade pelo Teste de Tukey. Ead: Epiderme adaxial; Pp: Parênquima paliádico; Pe: Parênquima esponjoso; Eab: Epiderme abaxial; Ef: Espessura do folíolo.

there was an increase of 56% in LII and 92% in SI with the highest dose.

3.3. Anatomical analyzes

The alterations observed in the leaves *B. virgilioides* were: disorganization of the mesophyll, with cells of different formats from the dose of 50 g i.a ha⁻¹ (Figure 3F) and with the doses 400, 800 and 1200 g i.a ha⁻¹ (Figures 3L, 3N and 3P), the tissues were totally destroyed with a twisted aspect; changes in the format of the chloroplasts mainly from the dose of 400 g i.a ha⁻¹; dark blue contents were detected inside the vacuoles of the cells of the epidermis and parenchyma, with the dose of 800 g i.a ha⁻¹; and accumulation of contents with reddish color in the palisade parenchyma, mainly at the dose of 200 g i.a ha⁻¹. After histochemical tests, the reaction to ferric chloride was observed only in the treatments with a blackish color in the palisade parenchyma, indicating the presence of general phenolic compounds. Hydrochloric vanillin did not indicate a positive reaction for tannins.

The micromorphometric analysis revealed that the plants exposed to glyphosate showed significant reductions in the thickness of the adaxial epidermis (Ead), palisade parenchyma (Pp) and spongy parenchyma (Sp). The most significant reductions were seen in the highest applied dose (1200 g i.a ha⁻¹). The parameters of thickness of the epidermis of the abaxial face (Eab) and thickness of the leaflet (Ef) did not show significant reductions (Table 2). Despite major changes in tissue structures, the reduction in the thickness of the leaflet was only 16% compared to the control (Table 2).

4. DISCUSSION

Exposure to glyphosate interfered with the physiological characteristics of *B. virgilioides* plants, changing all parameters of gas exchange evaluated, according to the increase in applied doses. The impacts caused by photosynthetic parameters are a consequence of the decrease in EPSPS and, consequently, by the accumulation of shikimic acid in plant tissues, which affects development (Gomes et al., 2017; Freitas-Silva et al., 2020). The decrease in the values of A , g_s and E , as observed in this study, may be partially related to the mode of action of glyphosate in stomatal cells, since the guard cells are directly affected by this herbicide, which can cause stomatal closure (Yannicari et al., 2012), reducing the flow of CO₂ available for photosynthesis, i.e. the lower the values of stomatal conductance the greater the resistance to CO₂ entry, causing significant effects on photosynthetic and transpiration rates, reducing carboxylative efficiency (Machado et al., 2010). Similar responses were observed by Batista et al. (2018), when analyzing the effects caused by glyphosate in the *Pouteria torta* species, where it was observed that these parameters decreased in response to the increase in glyphosate doses and the evaluation times, suggesting that the decrease in the photosynthetic process occurred, at least in part through stomatal limitations.

However, due to the decrease in stomatal conductance, it was expected that there would be a reduction in the C_i/C_a ratio, since the closure of the stomata limits the entry of CO₂, causing the consumption of CO₂ present in the mesophyll (C_i) naturally, thus decreasing the ratio (C_i/C_a) (Santos et

al., 2020). However, there was a significant increase in (C_i/C_a), indicating the accumulation of CO_2 in the substomatal cavity, a fact that shows that the effect was not caused only by stomatal limitations. According to Fernandes et al. (2015), considering that there is a constant concentration of external CO_2 (C_a), the increase in the ratio (C_i/C_a) occurs due to variations in the internal concentration (C_i). If there are increases in C_i , it means that the CO_2 which is reaching the mesophyll cells is not being fixed in the carboxylation phase of ribulose 1,5 bisphosphate carboxylase-oxygenase (Rubisco), probably due to lesions in the chloroplasts, reducing the photosynthetic rate. Freitas-Silva et al. (2020), when studying the responses of *Handroanthus chrysotrichus* to glyphosate, indicated that the increases in (C_i/C_a) may be due to biochemical limitations. These limitations are related to factors such as the limitation of the synthesis of the rubisco enzyme, and decreased chlorophyll synthesis promoted by the herbicide (Yannicari et al., 2012).

There were decreases in the Fv/Fm ratio, observed since the first evaluation, being more accentuated at higher doses, indicating that the applied doses damaged the photosynthetic apparatus. Values of this parameter in plants under non-stressed conditions range from 0.75 to 0.85, while decreases in these values are indicative of photoinhibition (Maxwell and Johnson, 2000; Silva et al., 2016). The reduction in the values referring to the $\Delta F / F_m'$ ratio, as well as the ETR and the increase in the NPQ, indicate that *B. virgilioides* suffered severe damage in the FSII. As a result, significant reductions were also found in the chlorophyll *a*, chlorophyll *b* and chlorophyll *a/b* ratio contents. Silva et al. (2016), also found reductions in chlorophyll content in the species *Caryocar brasiliense*, as did Batista et al. (2018), in *Pouteria torta* plants, and state that increased NPQ ameliorated the damage to the photosynthetic apparatus triggered by glyphosate in *Pouteria torta*, supported by reduced Fv/Fm, which indicates that damage to the structure of the thylakoids may have occurred, possibly due to photooxidative stress.

Changes in the content of chloroplast pigments caused increases in the electrolyte release rate (ERR), even at the lowest doses, indicating that glyphosate promoted membrane damage. It is believed that this is due to the fact that this herbicide stimulates the synthesis of the hormone ethylene, which, in turn,

stimulates the synthesis of phospholipid degradation enzymes, decreasing the stability of cell membranes (Yamada and Castro, 2007). Another explanation for this effect, is related to the increased formation of reactive oxygen species (ROS), because alterations in metabolic pathways caused by herbicides, are able to increase oxidative stress (Sharma et al., 2012), causing electrolyte leakage. Cruz et al. (2021), found that the overproduction of ROSs promoted lipid peroxidation in cell membranes of *E. uniflora* leaves exposed to glyphosate, which resulted in extravasation of cell contents, decreased photosynthetic rate, anatomical changes and visual symptoms of phytotoxicity - damage also observed in this study.

As a consequence of the physiological damage evidenced, the visual symptoms of intoxication of the plants exposed to glyphosate, were characterized by the presence of chlorosis and necrosis and the curling of some leaves. The phytotoxicity and severity indices were more expressive after 168 hours of application, and in the two highest doses (800 and 1200 g i.a ha⁻¹). Symptoms similar to these were also observed in other forest species subjected to glyphosate, such as *Caryocar brasiliense* (Silva et al., 2016), *Handroanthus chrysotrichus* (Freitas-Silva et al., 2020), and *Eugenia uniflora* L. (Cruz et al., 2021). Visual symptoms such as chlorosis and necrosis observed in *B. virgilioides* leaves may have occurred due to the overproduction of ROS, associated with anatomical changes such as protoplast retraction and cell collapse. These symptoms are common due to the initial interaction between the herbicide droplets and the plant's leaf surface (Lima et al., 2017; Freitas-Silva et al., 2020; Cruz et al., 2021).

In view of this, there was also damage to the anatomy of the leaf of *B. virgilioides*, there were changes in the organization of the mesophyll and in the format of the cells, being more expressive at higher doses, confirming that the effects are directly linked to the increase in doses. There was a significant reduction in thickness, in the adaxial epidermis and in the palisade and spongy parenchyma, and in higher doses the tissues were collapsed, the palisade and spongy parenchyma disorganized, with a twisted aspect. Santos et al. (2020), found similar symptoms in the *Cenostigma macrophyllum* species, mainly in the highest dose of glyphosate applied. For these authors, the reduction of observed leaf tissues may

have contributed to the deficiency of CO₂ diffusion in the mesophyll, a fact also observed in the present study.

The histochemical test confirmed the presence of phenolic compounds in *B. virgilioides*, and that the presence of these compounds was due to the application of the herbicide, as they were not found in the control treatment. It was found that these phenolic compounds are not tannins, as the hydrochloric vanillin did not indicate a positive reaction for them, requiring further studies with other reagents to identify which metabolic group these compounds belong to. According to Shaner et al. (2012), glyphosate probably caused these changes by acting on the shikimic acid route, which is responsible for the biosynthesis of aromatic amino acids such as tyrosine, tryptophan and phenylalanine, precursors of nitrogenous side products, which participate in the production of phenolic compounds.

5. CONCLUSIONS

The results indicated that glyphosate is phytotoxic to *B. virgilioides*, at the doses studied, as it promoted several physiological, anatomical and visual changes, which were more expressive with increasing doses. The changes observed in this study are possibly a consequence of the decrease in EPSPs, stomatal limitations, biochemical restrictions and overproduction of ROS, thus proving the sensitivity of *B. virgilioides* to glyphosate. Therefore, the observed responses may serve as a diagnostic tool for the effects of glyphosate on this species in vegetation fragments affected by the drifting of this herbicide, as its indiscriminate use is a risk to the Cerrado's biodiversity.

6. AUTHOR CONTRIBUTIONS

Ana Paula Augusta de Oliveira: Author of the work. Experiment execution, data analysis, interpretation of experimental data. Discussion and writing of the results in the article. Article Submission.

Ailton José Crispim-Filho: Collaboration in the experiment and statistical analysis.

Vanessa Ribeiro de Souza Santos: Assistance in the experiment and in reviewing the writing of the article.

Alan Carlos Costa: Co-orientation of the work. Assistance in the interpretation of physiological data analysis.

Kellen Lagares Ferreira Silva: Guidance and coordination of the work. Data design and analysis. Active participation in writing results and discussion. Final review. Article Submission.

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