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Physiological and Biochemical Changes in *Acacia Mangium* Seedlings under Glyphosate Application

Nadia da Silva Ramos[1,*] Sara Bezerra Bandeira[2], Flávia Barreira Gonçalves[1], Gessica Hashimoto de Medeiros[3], Marciane Cristina Dotto[5], David Ingsson Oliveira Andrade de[1], Rita de Cássia Moreira Rodrigues[5], Karolinne Silva Borges[1], Hallefy Elias Fernandes[1], Jéssica Bezerra Bandeira[3], Simone Pereira Teles[1], Márcio Silva Melo[1], Bruno SilvaMelo[1], Eduardo Andrea Lemus Erasmo[6]

[1]*Graduate Program in Plant Production, Federal University of Tocantins, Chácara 69-72 Rua Badejos, Lote 7 s/n - Jardim Sevilha, Gurupi, 77404-970, Tocantins, Brazil.*

[2]*Federal University of Lavras, Aquenta Sol, Lavras, 37200-000, Minas Gerais, Brazil.*

[3]*Graduate Program in Forestry and Environmental Sciences, Federal University of Tocantins, Chácara 69-72 Rua Badejos, Lote 7 s/n, Gurupi, 77404-970, Tocantins, Brazil.*

[4]*Federal Institute of Tocantins, Rodovia To-040, Km 349, Loteamento Rio Palmeira, Lote 1 0 Zona Rural, Dianópolis, 77300-000, Tocantins, Brazil.*

[5]*Graduate Program in Agroenergy, Federal University of Tocantins, Avenida NS-15, Quadra 109, Norte, s / n - North Master Plan, Palmas, 77001-090, Tocantins, Brazil.*

[6]*Federal University of Tocantins, Block 109 North, Avenida NS-15, ALCNO-14 North Master Plan, Palmas, 77001-090, Tocantins, Brazil.*

[*]Corresponding Author: ramos.n@bol.com.br (Nadia da Silva Ramos)

Abstract

With the high growth of planted forests, some investors seek to implement these crops with high production and a relatively low implementation cost. In the state of Tocantins, these activities have been increasing without any kind of planning. The application of glyphosate has been one of the ways found by producers to reduce the cost of herbicides because it is one of the most-used herbicides in the world, due to its wide spectrum of action, however, in some tree species, there is still little knowledge about the effect on physiology, biochemistry, and growth. The objective of this research was to evaluate the physiological, biochemical and morphological changes of Acacia mangium under the application of glyphosate doses. The young plants of Acacia Mangium received 5 treatments corresponding to herbicide doses (0, 180, 360, 540 and 720 g.e.a.L^{-1}glyphosate) in 6 replications. We evaluated increments in diameter, height, dry mass of the stem, leaves and root, intoxication, and enzymatic changes antioxidant (peroxidase ascorbate (APX)) and liquid assimilation (A), stomatal conductance (gs), internal carbon concentration (Ci), transpiration (E), water use efficiency (WUE) and carboxylation efficiency (A/Ci). After glyphosate application, it was found that the doses of (540 g.e.a.L^{-1}) and (720 g.e.a.L^{-1}) caused a decrease in the dry mass of the stem, leaves, roots and root volume, and decrease dwell of leaf area to dose (540 g.e.a.L^{-1}) and the increase of leaf area to the dose (720 g.e.a.L^{-1}) which is justified by the overgrowth of branches at this dose. In the determination of APX, it observed a trend of increase with the increase of glyphosate doses in the two evaluated periods. Regarding phytotoxicity, the doses of (180 and 360 g.e.a.L^{-1}) presented 100% intoxication with mild injuries and in the other doses (540 and 720 g.e.a.L^{-1}) 20% of moderate injuries and 80% of mild injuries at days after application. For net assimilation (A) the doses of (540 and 720 g.e.a.L^{-1}) showed a decrease in the rate when compared to the control, similar to the behavior of Carboxylation Efficiency (A/Ci). Plants treated with the highest doses 540 and 720 g.e.a.L^{-1} showed the lowest transpiration values throughout the evaluation period and there was a negative effect with the use of the highest dose (720 g.e.a.L^{-1}), with an increase in dry matter production per unit of water, affecting the efficiency of water use.

Keywords: Arboreal species, Antioxidant enzymes, Photosynthesis.

1. INTRODUCTION

The species *Acacia mangium* is of great commercial value and is highly cultivated especially for primary energy [1] (VALE et al., 2000) and cellulose [2]. It has relatively fast growth and an average life of 40 years [3] (VEIGA et al., 2000), good development in several regions of the country, not being demanding in nutrient-rich soils [4].

The use of chemical weed control in forest species is of paramount importance, however, there are still few studies that report the effects of the stress suffered by them when exposed to glyphosate drift [5]. However, it is known that this herbicide not selective for forest species can cause some metabolic changes being one of the most relevant the accumulation of shikimic acid and formation and/or activation of oxidative reactive enzymes (ORE).

The accumulation of shikimic acid is directly linked to inhibition of the enzyme EPSPS (5-enolpiruvilchiquimate-3-phosphate synthase), caused by the herbicide glyphosate, which can prevent the synthesis of the three essential amino acids, tryptophan, phenylamine, and tyrosine, causing changes in photosynthesis, respiration, and transpiration [6] (YAMADA et al., 2007), one of the causes of oxidative stress.

According to Yamada & Castro (2007) [6] the plant contaminated with glyphosate has decreased growth of the aerial part and root system in addition to the loss of resistance against diseases, this even with low doses such as 3 mL ha^{-1} of the commercial product. Authors such as [6, 7] (Galli & Montezuma, 2005; Yamada & Castro, 2007), warn that glyphosate if used improperly, can cause phytotoxicity, or even lead to the death of plants.

Tuffi Santos et al., (2005[a]) [8] evaluating leaf growth and morphoanatomy in eucalyptus, found that eucalyptus plants in contact with glyphosate showed their growth compromised, and the amplitude of dose-dependent growth reduction glyphosate to which the plant was submitted.

According to Silva et. al., (2016) [9], glyphosate doses caused variations in photosynthetic elements in Pequi (Caryocar brasiliense) plants, causing reductions in gas exchange, photochemical efficiency, and chloroplastidic pigment content, basically when herbicide doses (250, 500, 1,000 and 1,500 g ha^{-1}).

Because of the above, this study aims to evaluate the physiological, biochemical and morphological alterations of glyphosate in *Acacia mangium* plants.

2. MATERIALS AND METHODS

This study was carried out at the experimental station of the Federal University of Tocantins (UFT), Gurupi University Campus, located in the southern region of the state of Tocantins at 11◦ 43' S and 49◦ 04' W, at 280 m altitudes.

Polyethylene bags with a volume of 10L were used for which the plants were transplanted with a height of 30cm. When the plants were 60 cm high, applications were made with a pressurized carbon dioxide costal spray and equipped with nozzle tips (XR 110.02) with a syrup volume of 200 L/ha^{-1} and the constant pressure of 35 kgf/cm^2. The application was carried out to reach the entire canopy of the plant.

The experiment was installed in a randomized block design with 5 treatments corresponding to 4 doses of glyphosate plus the control without application (0, 180, 360, 540 and 720 g.e.a L^{-1}) and 6 replicates, using 6 plants per repetition.

2.1. Biometric measurements and biomass

After herbicide application, observations were made at 7, 14, 21, 28 and 35 days after application (DAA), morphological changes in the aerial part of the plants, with the naked eye, considering the size of the plant, leaf color, and general development, as well as the plant intoxication (PI) about the control using a scale of notes from 0 to 100%, where: 0% for the absence of symptoms and 100% for total plant death [10].

The evaluations of plant height (H) and stem diameter (D) were performed before application and at 75 after application (DAA), where all plants were measured. At 75 DAA, the leaf area of the plants was determined by the destructive method with the aid of an "Area Meter" leaf area meter (LI-3000 LIQUOR model), after which the leaves, stems, and roots were packed in paper bags and dried in a greenhouse at 69◦C until weight, with subsequent determination of dry mass (DM).

2.2. Extraction and activity of the antioxidant enzyme

Samples of plant leaves were collected at 10 and 20 days after herbicide application to determine peroxidase ascorbate activity. The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was determined by monitoring the oxidation of guaiacol at 470 nm with modifications. All these analyses were performed in triplicate.

2.3. Gas exchange analysis

Initially, the photosynthesis saturation curve (light curve) was measured using photosynthetically active radiations – RAF of 0, 25, 50, 100, 250, 500, 800, 1000, 1200, 1500, 1800, 2000, 2200 mol m^{-2-1}, to determine the best radiation for the realization of the readings. The evaluations were performed at 6, 12, 18, 24 and 30 days after herbicide application (DAA). Data were collected from 9:00 a.m. to 11:00 a.m., in the fourth leaf counting from the meristem of the plant, and 6 plants were sampled by treatment. The evaluations of gas exchange were performed based on the measurement of the assimilation rate of CO_2 (A) (μmol m^{-2-1}), transpiration (E) (mmol of H2O m^{-2-1}), stomatal conductance (gs) (mmol m^{-2-1}), the internal concentration of CO_2 (Ci) (μmol m^{-2-1}). In possession of these data, the efficiency in water use (EWU) (mmol CO_2 mol^{-1} H_2O) (A/E) [(μmol m^{-2-1}) (mmol H_2O m^{-2-1})-1] and the instantaneous efficiency of carboxylation (EiC) (A/Ci) [(μmol m^{-2-1}) (μmol mol^{-1})-1] (Machado et al. 2005 Melo et al. 2010) [11, 12]. All these parameters evaluated were obtained directly under ambient CO_2 conditions using a portable infrared gas analyzer (IRGA -

Infra-Red Gas Analyser, model LI-6400 XT, Li-cor, inc. Lincoln, USA).

Morphological, biochemical and physiological variables were submitted to variance analysis using the F test and for the Tukey test, where the significance level less than or equal to 5% was adopted using the Software SISVAR program [13].

3. RESULTS

All glyphosate doses caused a reduction in the growth of acacia seedlings, expressed in the decrease in height increment when compared to the control. However, the percentage of height increment obtained in the treatments with doses 180 and 360 g.e.a. L^{-1} did not differ from each other, showing an increase of 33.33% in both treatments. The smallest increments were observed in the treatments with doses of 540 and 720 g.e.a. L^{-1} (3.33% and 1.66%, respectively). Thus, it can be observed that there was a reduction in height with the increase of the tested herbicide dose. Treatments 180 and 360 g.e.a. L^{-1} showed an increase in 50% lower than the control.

The increase in stem diameter of *A. mangium* seedlings was also influenced by glyphosate application. The results were similar to those obtained for height, where the control showed a higher percentage of the mean increment (47.66%), differing statistically from the others. The treatments with doses of 360, 540 and 720 g.e.a.L^{-1} did not differ statistically from each other, generally presenting the lowest values observed, of 24%, 21.66%, and 18% respectively.

Evaluating the phytotoxicity levels caused by glyphosate doses in *A. mangium* plants, it was observed that at 7 days after application, the doses applied (180 and 360 g. e.a.L^{-1}) caused in 100% of the individuals significant levels of injuries considered mild injuries at doses (540 and 720 g. e.a.L^{-1}) caused about 20% of plants of moderate injuries and 80% of individuals with minor injuries. At 14 and 21 days the injuries of the doses of (180 and 360 g. e.a.L^{-1}) continued in 100% of the individuals with only injuries considered mild, however in the doses (540 and 720 g. e.a.L^{-1}) there was an increase in phytotoxicity of 5% of the individuals with injuries (540 and 720 g. e.a.L^{-1}) there was an increase in phytotoxicity of 5% of the individuals with injuries severe and 95% with moderate injuries with reduced growth.

At 28 and 35 days after application, it was noticed that at doses of (180 and 360 g. e.a.L^{-1}) there was no increase in phytotoxicity, but a 5% reduction in phytotoxic effects of the herbicide, presenting a gradual recovery of plants in about 50% of the individuals, however, there was still 50% of individuals with mild injuries, in doses (540 and 720 g. e.a.L^{-1}) there was a decrease in phytotoxicity of 10%, that is, from severe to moderate injuries, with reduced growth and diameter.

The dry mass of the stem (DMS), showed a significant difference in the doses of 180 and 360 g.e.a. L^{-1}, causing an increase of 63.28% and 53.72% about the control, the dry mass of the root (DMR), occurred a decrease in doses 360, 540 and 720 g.e.a. L^{-1}, that is, loss, 26.22%, 44.85%, and 57% reduction when compared to the control. The values of (DML) dry mass of leaves indicate an impact of glyphosate at the highest doses 360, 540 and 720 g.e.a. L^{-1}, reducing the dry mass of leaves by 31.10%,44.4%, and 59.35% respectively.

Root volume (RV) values showed significant differences between treatments at doses of 360, 540 and 720 g.e.a.L^{-1}, increasing their volume by 24.99%, 17.64% and 27.95% relatively (Table 1). A similar result was observed in the leaf area, where doses of 360 and 540 g.e.a.L^{-1} promoted reductions in the order of 51.83% and 36.26%. The 720 g.e.a.L^{-1} dose increased by 7.6%, probably due to the appearance of overgrowth.

Table 1. Dry mass of the stem (DMS), dry mass of the leaf (DML), dry mass of the root (DMR), root volume (VR) and leaf area of *Acacia mangium* seedlings with the application of the herbicide glyphosate in four doses: T1 - Control (without application); T2 (180 g.e.a.L^{-1}), T3(360 g.e.a.L^{-1}), T4 (540 g.e.a.L^{-1}) and T5 (720 g.e.a.L^{-1}) at 75 days after application (DAA), in Gurupi, TO, 2016**.**

Treatments	DMS	DML	DMR	VR (cm³)	Foliar Area (cm²)
Witness	15,69 b	20,00 a	36,07 a	45,33 b	11940,99
180 (g.e.a. L^{-1})	25,62 a	18,34 a	38,00 a	44,33 b	11817,30
360 (g.e.a. L^{-1})	24,12 a	13,78 b	26,61 b	56,66 a	575089
540 (g.e.a. L^{-1})	14,94 b	11,12 b	19,89 c	53,33 a	761033
720 (g.e.a. L^{-1})	11,72 b	8,13 b	15,51 c	58,00 a	1285431
CV (%)	12,36	17,03	10,8	8,14	29,28

Averages followed by the same lowercase letter in the column do not differ by the Scott Knott test at 5% probability.

Analyzing the enzyme peroxidase of ascorbate (APX) in seedlings of *Acacia mangium* at 10 and 20 days after application (DAA) as a function of glyphosate herbicide concentration. It was observed that the APX showed a tendency to increase with the increase of glyphosate doses in the two evaluated periods (Figure 1).

At 10 DAA, in the control, the APX value corresponded to 172.2433 mml, while at doses of 180, 360, 540 and 720 g.e.a. L^{-1} the values corresponded to 249.5349 mml, 264.8271 mml, 364.7114 mml, and 522.2707 mml respectively. Already at 20 DAA, it was observed that the value observed in the control (268.9937 mml) was higher than that observed at the dose of 180 (160.3201 mml), however at doses of 360, 540 and 720 g.e.a. L^{-1}, there was an increase in the values of APX, with values of 365.6616 mml, 403.7129 mml, and 641.1397 mml.

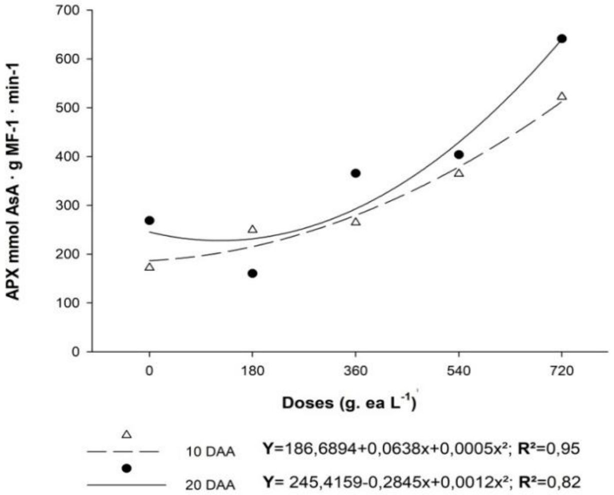

Figure 1: Ascorbate peroxidase (APX) in Seedlings of *Acacia mangium* with 10 and 20 days after application (DAA) of glyphosate herbicide in four doses (180, 360, 540 and 720 g.e.a.L^{-1}) and the control (without application), in Gurupi, TO, 2016.

CO_2 assimilation (A) showed similar values between the control (4.63 μmol. m^{-2-1}) and the dose of 360 g.e.a L^{-1} (4.71μmol. m^{-2-1}) at 6DAA, while for the other days of evaluation, a statistical difference was observed, with a reduction of CO_2 assimilation in plants application of the herbicide. At 18 and 30 DAA, no statistical difference was observed between the control (7.34 and 7.20 μmol. m^{-2-1}, for the respective days) and the dose of 180 g.e.a L^{-1} (7.11 and 7.80 μmol. m^{-2-1}, respectively, for each day).

The highest doses of 540 g.e.a L^{-1} and 720 g.e.a L^{-1} hurt the plants of *Acacia mangium*, with reduced CO_2 assimilation, differing statistically from the control. There was a decrease of approximately 67, 73 and 74% for (A), respectively, at 12, 18 and 24 DAA at the dose of 540 g.e.a L^{-1}. E of 73, 88 and 89% at the dose of 720 g.e.a L^{-1} at 12, 18 and 24 DAA of glyphosate, about the witness.

From the 24 DAA, it was observed that the plants submitted to concentrations of 360, 540 and 720 presented recovery with an increase in the CO_2 assimilation rate.

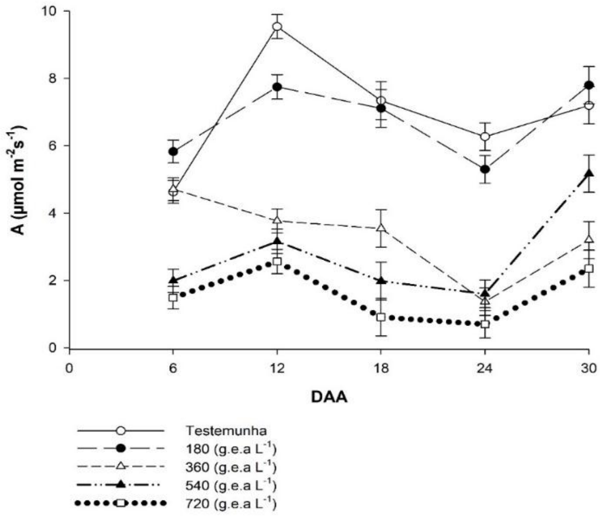

Figure 2- Net assimilation of CO_2 (A) in Seedlings of *Acacia mangium*, submitted to different doses of glyphosate. **Gurupi - TO, 2016.**

Regarding transpiration, significant differences were observed at 5% between the control, and all doses of glyphosate tested 180, 360, 540 and 720 g.e.a. L^{-1} throughout the evaluation period. Plants treated with the lowest dose (180 g.e.a. L^{-1}) showed recovery, with values close to those observed for the control. Plants treated with the highest doses of 540 and 720 g.e.a. L^{-1} showed the lowest transpiration values throughout the evaluation period.

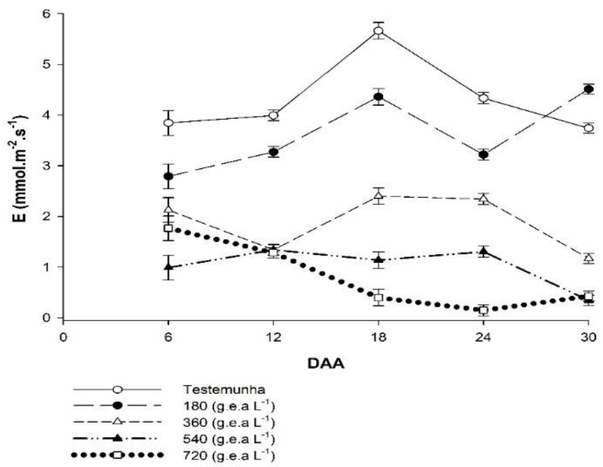

Figure 3- Transpiration (E) in Seedlings of *Acacia mangium*, submitted to different doses of glyphosate. Gurupi - TO, 2016.

Regarding the Internal carbon concentration (Ci), there was no statistical difference in any of the doses 180, 360, 540 and 720 g.e.a. L^{-1} of glyphosate, about the control, in all evaluated days. In general, it was observed that higher concentrations of glyphosate affected the physiological characteristics evaluated over time with the application of doses higher than 180 g.e.a. L^{-1}.

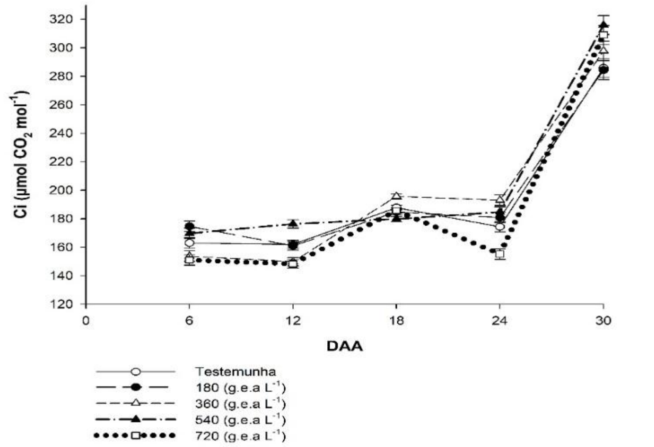

Figure 4- Internal carbon (Ci) in *Acacia mangium* seedlings, submitted to different doses of glyphosate. Gurupi - TO, 2016.

The efficiency of water use is characterized as the amount of water needed for the production of a certain amount of dry matter for a crop. Throughout the experimental period, it was observed that there was a variation in the efficiency of water use in plants, and at 6, 12 and 18 DAA the doses of 180, 360 and 540 g.e.a. L^{-1} presented values close to those observed for the control.

There was a negative effect with the use of the highest dose (720 g.e.a. L^{-1}), with an increase in dry matter production per unit of transpired water, affecting the efficiency of water use.

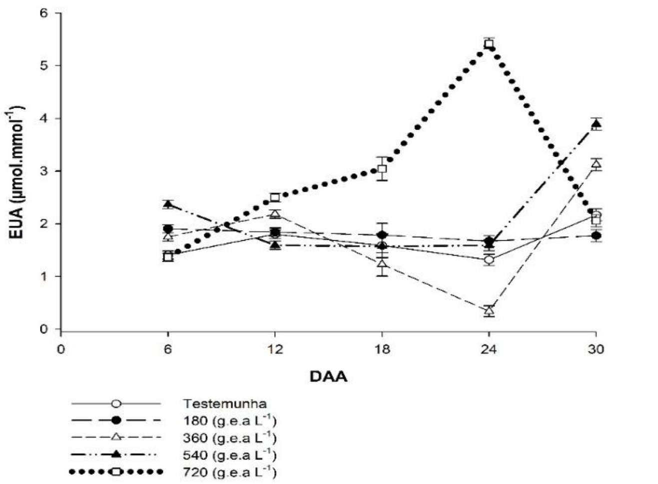

Figure 5- Water Use Efficiency (WUE) in *Acacia mangium* seedlings, submitted to different doses of glyphosate. Gurupi - TO, 2016.

The behavior of Carboxylation Efficiency (*A/Ci*) was similar to that observed for liquid CO_2 assimilation. At 6 DAA, no significant differences were observed between the control, the dose of 180 and 360 g.e.a.L^{-1}. In the other days of evaluation, a reduction of carboxylation efficiency values was observed for the dose of 360 g.e.a.L^{-1}.

The highest doses of 540 g e.a L^{-1} and 720 g e.a L^{-1} showed a negative effect for *Acacia mangium* plants, with decreased CO_2 assimilation, differing statistically from the control.

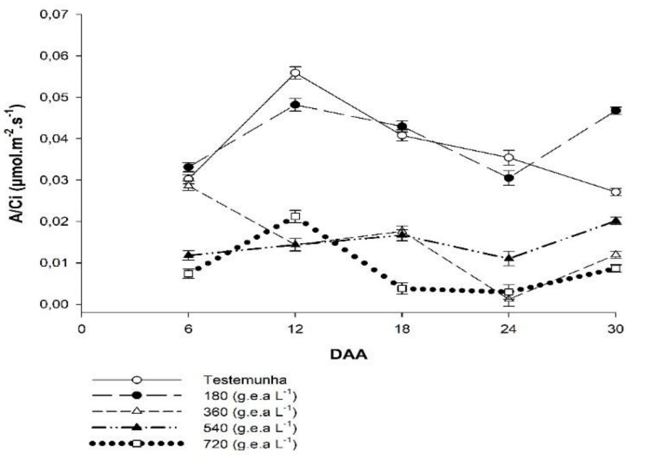

Figure 6- Efficiency of Carboxylation (*A/Ci*) in Seedlings of *Acacia mangium*, submitted to different doses of glyphosate. Gurupi - TO, 2016.

4. DISCUSSION

Glyphosate is one of the most widely used herbicides in the world, EPSPS enzyme inhibitor, through the metabolic pathway of shikimic acid, can cause biochemical damage in plants. The excess of this herbicide in some vegetables can cause oxidative stress, due to the increased production of Reactive Oxygen Species (ROS) considered highly toxic. Reactive oxygen species (ROS), represented by superoxide radicals (O_2•-), hydrogen peroxide H_2O_2) and hydroxyl radical (OH•) promote damage to proteins and lipids of plant cells membranes [14].

To avoid oxidative damage, plants present antioxidant defenses involving enzymes, such as ascorbate peroxidase (APX) [15]. The action of glyphosate interrupts the carbon cycle in chloroplasts, one of the main sites that form ROS, as well as mitochondria and peroxisomes. These enzymes participate in the modulation of the H_2O_2 level in chloroplasts, mitochondria, cytosol, and peroxisomes [16]. Once they dismute O_2-, they act indirectly in reducing the risk of oh deformation from O_2- [17]. An increase in the activity of the enzyme peroxidase can be considered a protective action, as they would neutralize the OREs in water and molecular oxygen, thus avoiding lipid peroxidation.

APX is one of the main enzymes responsible for the detoxification process of H_2O_2 in plants and can directly dismute H_2O_2 or oxidize substrates, forming non-toxic products to the cell [18]. In this work, APX showed an increase in enzymatic activity according to the increase in glyphosate doses, these peroxidases are responsible for maintaining control of both production and removal of OH-radicals. This is because APX is the first inactivated enzyme after a short period of light, and even in the absence of the herbicide, the addition of H_2O_2, in the dark, inactivates APX [19] (HOSSAIN; ASADA, 1984), i.e. as there was no lack of light it continued to increase.

Peroxidases are believed to play a substantial role in eliminating herbicide-induced cell damage. Because it is predominantly cytosolic and organellar [20] (FOYER; NOCTOR, 2000), APX uses ascorbate as its specific electron donor to eliminate H_2O_2. The greatest induction of oxidative stress was also demonstrated by the action of xenobiotics, including paraquat, glyphosate, and norflurason [21]. However, in corn and beans particularly, not all herbicides induce oxidative stress [22]. It is believed that changes in the activity of antioxidant enzymes exposed to herbicide application and evaluated over time are directly linked to the suppression of oxidative stress by eliminating ORE produced by herbicide application.

When treated with higher herbicide concentrations, the plants showed a reduction in net carbon assimilation (A), being considered a negative effect, because according to Yamada et al. (2007) [6], the photosynthetic rate is directly linked to the accumulation of dry matter on the part of the plant and this reduction for a prolonged period leads to decreases in crop productivity. Glyphosate in high doses decreases the photosynthetic efficiency of plants, as it concentrates a quantity of shikimic acid due to inhibition of the Enzyme EPSPS, presenting as a strong carbon drain in the Calvin cycle, stimulated by the deviation of the erythosis-4-phosphate that would be taken in the regeneration of ribulose [23].

The non-regeneration of ribulose and photosynthetic reduction is related to photorespiration that occurs due to the double activity exerted by the enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO). RuBisCO, present in chloroplasts, besides exerting the activity of carboxylase, which initiates the Calvin cycle, is also capable of exerting oxygenase activity, having, in both cases, RuBP as substrate, hence its name "ribulose-1,5-biphosphate carboxylase/oxygenase". The carboxylase or oxygenase activity of RuBisCO depends on the concentrations of CO_2 and O_2 in the stroma [24].

In the concentration of 180 g and L^{-1}, it verified a positive effect, stimulating the increase of photosynthesis, stomatal conductance and consequently transpiration. This effect was verified by Cedergreen and Olesen (2010) [25], observing that the use of small concentrations of glyphosate can cause stimulation in photosynthesis, characterizing the phenomenon of hormesis.

The physiological stress caused by glyphosate induces ethylene production, which can destroy membranes, inhibit chlorophyll and reduce stomatic closure [6]. This production is due to leaf abscission that can be delayed by auxin or exogenous cytokinin. Plants treated with doses of 360, 540 and 720 $g.e.a.L^{-1}$ also showed lower values of somatic conduction, and consequently a decrease in transpiration. Gs has its regulation is by plant hormones such as abscisic acid (manufactured by leaves, roots, and stem), cytokinins (in the roots) and gibberellins (meristems, new leaves, and fruits), facilitating the transport of CO_2 [25].

The efficiency of water use for the highest dose (720 $g.e.a.L^{-1}$) at 18 and 24 DAA after application increased, where there was a decrease in transpiration and photosynthesis, and a reduction in stomatal conductance. The decreases observed in WUE, notably at the time of 10:00 am to 11:00 am, are reflections of the increases observed in the CO_2 assimilation rate and transpiration of *A. mangium* plants. Shimazaki et al. (2007) [26], point out that the maintenance of the photosynthetic process is functionally linked to a certain stomatal conductance. The efficiency of carboxylation is given by the relationship between photosynthesis and internal carbon concentration, also regulated by stomatal conductance. It was verified that the measure that increased the net assimilation of carbon (A), there was an increase in the opening of the stomata and consequently of the (A/Ci).

At the lowest dose 180 g.e.a. L^{-1} the plant had an increase in the efficiency of carboxylation, where it can be said that it has a close relationship with the intracellular concentration of CO_2 and the assimilation rate of carbon dioxide [11]. In this sense, the increase in the instantaneous efficiency of carboxylation in the present study is mainly due to the increases recorded in the internal concentration of carbon dioxide and the gains in the assimilation rate (A).

5. CONCLUSION

Glyphosate reduced dry mass production, leaf area, height and stem diameter increment at the highest doses of 360, 540 and 720 g and. to L^{-1}. Doses of 360, 540 and 720 g e. to L^{-1} of glyphosate reduced liquid assimilation, stomatal conductance, and transpiration.

The lowest dose of glyphosate of 180 g e. to L^{-1} stimulated the growth of the Plant of *A mangium*, in 33.3%. *A. mangium* showed little variation in water use and carboxylation efficiency when submitted to herbicide action. The species *A. mangium* presented an antioxidant enzymatic defense system (APX) in combat against (ROS).

APX showed higher activity at the highest doses 360, 540 and 720 $g.e.a.L^{-1}$ respectively from 300 to 600 mmol AsA. g $MF^{-1}.min^{-1}$ exhibiting compensatory mechanisms to defend oxidative stress.

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