

# Glyphosate excessive use chronically disrupts the shikimate pathway and can affect photosynthesis and yield in citrus trees

Rodrigo Martinelli<sup>a,\*</sup>, Luiz Renato Rufino Jr.<sup>b</sup>, Ana Caroline de Melo<sup>b</sup>, Ricardo Alcántara-de la Cruz<sup>c</sup>, Maria Fátima das Graças Fernandes da Silva<sup>d</sup>, Jefferson Rangel da Silva<sup>a</sup>, Rodrigo Marcelli Boaretto<sup>a</sup>, Patricia Andrea Monquero<sup>b</sup>, Dirceu Mattos Jr.<sup>a</sup>, Fernando Alves de Azevedo<sup>a</sup>

<sup>a</sup> Agronomic Institute (IAC), Sylvio Moreira Citrus Center, Cordeirópolis, São Paulo, Brazil

<sup>b</sup> Federal University of São Carlos (UFSCar), Araras, São Paulo, Brazil

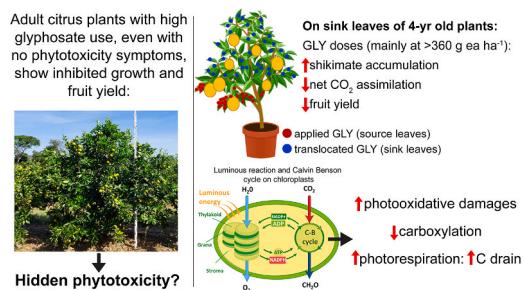
<sup>c</sup> Federal University of São Carlos (UFSCar), Buri, São Paulo, Brazil

<sup>d</sup> Federal University of São Carlos (UFSCar), São Carlos, São Paulo, Brazil

## HIGHLIGHTS

- Glyphosate at >360 g ae ha<sup>-1</sup> accumulates shikimate and affects citrus photosynthesis.
- Damages were persistent over time and were enhanced after a second application.
- Damages were observed even as the phytotoxicity symptoms were not visible.
- Photooxidative damages and a partial carbon drain that consequently reduces the citrus fruit yield.
- First report to demonstrate the chronic damage of glyphosate on citrus trees.

## GRAPHICAL ABSTRACT



## ARTICLE INFO

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## ABSTRACT

Glyphosate excessive use is reported in Brazilian citrus orchards, whereas there is speculation about its consequences and the published studies are contradictory and inconclusive. This study aimed to describe the possible harmful effects by simulating glyphosate drift directly to the leaves of ~4-yr-old citrus plants. As major results, glyphosate doses >360 g ae ha<sup>-1</sup> increased the shikimate accumulation in leaves (up to 2.3-times above control), which was increased after a second glyphosate application (up to 3.5-times above control), even after a 240-d interval. Interestingly, shikimate accumulation was occasionally related to a dose-response of the herbicide at specific times; however, the doses had their accumulation peak on determined dates. These accumulations were directly correlated to reduced net photosynthesis even months after the glyphosate sprays. Quantum productivity based on electron transport through the photosystem II and apparent electron transport reductions up to 17% were also observed during the entire experiment course. Similarly, quantum productivity based on CO<sub>2</sub> assimilation of glyphosate sprayed leaves decreased up to four times compared to the control after the second application. Glyphosate doses >360 g ae ha<sup>-1</sup> increased stomatal conductance and transpiration as the carboxylation efficiency decreased, evidencing a carbon drainage in the Calvin-Benson cycle. These metabolic

\* Corresponding author. Agronomic Institute (IAC), Sylvio Moreira Citriculture Center, Cordeirópolis, São Paulo, Brazil..

E-mail addresses: [martinelli@ccsm.br](mailto:martinelli@ccsm.br), [rdr.g.martinelli@gmail.com](mailto:rdr.g.martinelli@gmail.com) (R. Martinelli).

and physiological disturbances suggest possible photooxidative damage and an increase in photorespiration, which may be a mitigation strategy by the citrus plants to glyphosate effects, by the cost of reducing the citrus fruit yield (up to 57%). It is concluded that glyphosate phytotoxicity damages citrus plants over time due to chronic disturbances in the shikimate pathway and photosynthesis, even when there are no symptoms. This study is the first report to demonstrate how glyphosate damages citrus trees beyond the shikimate pathway.

## 1. Introduction

Brazil is responsible for 15% (19.6 mi t) of world citrus production, ranking second only behind China, accounting for 25% (33.2 mi t) (FAO – Food and Agriculture Organization of the United Nations, 2022); therefore, citrus farming is of considerable economic and social importance (Azevedo et al., 2020). Weeds are the main biotic factor that directly or indirectly affects both the citrus fruit yield and quality depending on the age of the orchard, becoming responsible for economic losses that can exceed 50% (Martinelli et al., 2017; Alcántara-de la Cruz et al., 2020).

Although various programs, including chemical and non-chemical alternatives, have been proposed for sustainable weed management in citrus orchards, most Brazilian citrus growers prefer chemical control based mainly on glyphosate-based herbicides (Martinelli et al., 2017; Azevedo et al., 2020). Glyphosate [N-(phosphonomethyl)glycine] is a non-selective herbicide that is translocated mainly to metabolic sinks and growth meristems, which can be distant from the application site. The high mobility of the molecule in phloem and the slow action allows the herbicide to move throughout the plant to hamper meristems, making it effective even for controlling perennial weeds (Duke, 2020). Glyphosate was launched in 1974, and the volume applied worldwide increased significantly (>100 times), mainly due to the patent loss in 2000, low cost (when compared to other herbicides), increased application doses to control herbicide-resistant weeds, broad adoption of transgenic glyphosate-resistant crops (e.g., Round-up Ready®), and to new usage patterns, such as pre-harvest, desiccation of cultivated or non-cultivated areas (Myers et al., 2016).

Herbicide applications in citrus crops are usually directed to the orchards intra-row area (under the canopy of the trees) and throughout the orchards inter-row. A survey on weed management conducted with citrus growers from several Brazilian producing regions revealed that 98% use glyphosate, of which 36% exclusively use this herbicide (Martinelli et al., 2022). As well, 73% use >1000 g acid equivalent (ae) ha<sup>-1</sup>: 56% between 1000 and 1500 g ae ha<sup>-1</sup>, 6% from 1500 to 2000 g ae ha<sup>-1</sup>, and 11% > 2000 g ae ha<sup>-1</sup>. Furthermore, 11% spray once per year, 47% spray twice, 22% spray three times, 9% spray four times, and 11% spray five or more times.

There is still speculation about the consequences of using glyphosate on citrus orchards. The published studies are contradictory and inconclusive, with more recent studies showing little glyphosate phytotoxicity to citrus or even a transient effect (Gravina et al., 2009, 2012; Matallo et al., 2010). However, some older studies reported glyphosate-induced effects in citrus trees by visual symptoms of its phytoinhibition, such as leaf chlorosis, leaf drop, and shoot malformation (Tucker, 1977), which can remain for two years in the citrus plants (Toth and Morrison, 1977). Likewise, fruit drop was also reported on diverse citrus varieties, depending on a high dosage of glyphosate applied directly to fruit in an advanced stage of development (Erickson, 1996). Furthermore, a recent field study showed that the damages caused by glyphosate when misused in citrus crops surpass its benefits as an herbicide (Martinelli et al., 2022).

The glyphosate mechanism of action is unique, first acting on the shikimic acid pathway, being able to inhibit the 5-enolpiruvil-shikimate-3-phosphate-synthase (EPSPS) by occupying the binding site of the phosphoenolpyruvate (PEP) (Amrhein et al., 1980). Glyphosate's effects are broad, starting with the accumulation of shikimate and inhibiting the formation of chorismate, a precursor of salicylic acid (plant defense

hormone), and three aromatic amino acids (tryptophan, tyrosine, and phenylalanine) (Amrhein et al., 1980; Gomes et al., 2014), which are precursors of a wide variety of aromatic compounds. Disruption in the production of these aromatic substances affects the biosynthesis of flavonoids, phenolic compounds, monolignol polymerization, lignin synthesis, and other secondary metabolites, which have crucial roles in plant development, such as growth, reproduction, and defense mechanisms (Haslam, 1993; Herrmann, 1995). These compounds are responsible for ~30% of the carbon fixed by photosynthesis and ~30% of the dry weight of the plant, i.e., glyphosate also deregulates the carbon flow and the functioning of other biochemical pathways (Maeda and Dudareva, 2012; Orcaray et al., 2012).

Based on the above, it was hypothesized that the increased use of glyphosate impairs the development of citrus trees through shikimate accumulation over time, which affects photosynthesis and consequently reduces fruit yield. Thus, this study aimed to determine the shikimate accumulation over time, the effects on photosynthesis and fruit yield, and their relationship in the citrus plants caused by glyphosate applications directly on the leaves, simulating herbicide drift and direct contact, which are expected in field orchards.

## 2. Material and methods

The experiment was conducted in a greenhouse of the Agronomic Institute (IAC), Sylvio Moreira Citriculture Center, Cordeirópolis, State of São Paulo, Brazil (22°27'37.8"S and 47°24'02.8"W). A completely randomized experimental design was used, composed of seven glyphosate doses (0, 45, 90, 360, 720, 1440, 2880 g ae ha<sup>-1</sup>) and three replications. Each experimental unit consisted of a 40 L pot with a citrus plant.

Valencia orange [*Citrus sinensis* (L.) Osbeck] seedlings grafted on Rangpur lime (*C. limonia* Osbeck) at 24-month-old were transplanted into pots containing 80% pine bark, 15% vermiculite, and 5% substrate in August/2018. The plants were irrigated at 70% field capacity and fertilized with a complete solution of macro and micronutrients (Hippler et al., 2015).

Seedlings were grown for 19 months after transplant. Then, two glyphosate applications (RoundUp Original®, 356 g ae L<sup>-1</sup>, Monsanto, São Paulo, São Paulo State, Brazil) were performed in March/2020 (App. #1) and November/2020 (App. #2) (240 d interval). The applications were calibrated to deliver 200 L ha<sup>-1</sup> at 2.0 bar using a backpack CO<sub>2</sub> sprayer (Névoa Comercial, Campinas, São Paulo State, Brazil) with a Teejet® Extended Range (XR) 11002 VS nozzle (Teejet Technologies South America, Cotia, São Paulo State, Brazil). Herbicide drift was simulated by applying the fixed value of 0.0075% (v/v) of the recommended spray volume (15 mL plant<sup>-1</sup>). The application was directed to the lower third of the plant canopy (source leaves). The middle and upper thirds were protected with high-density polyethylene plastic and removed 2 h after applications. At the time of App.#1, plants were 44 months old, with a canopy volume of ~1.2 m<sup>3</sup>, to simulate the conditions of mature plants in the field, which have most of their stems fully lignified, limiting the absorption of glyphosate exclusively by the leaves.

### 2.1. Chemicals and reagents

Hydrochloric acid 37% (purity: ≥99%), periodic acid (purity: ≥99%), sodium meta periodate (purity: ≥98%), sodium hydroxide (purity: ≥99%), and sodium sulfite (purity: ≥98%) were acquired from

Synth (Diadema, São Paulo State, Brazil). Shikimic acid (Shikimate, purity: ≥99%) was bought from Sigma-Aldrich (São Paulo, São Paulo State, Brazil).

## 2.2. Quantification of shikimate concentration in leaves

Shikimate (*Shk*) concentrations were determined in six recent mature leaves collected from the mid-third of the citrus plants (sink leaves) at 0, 1, 7, 15, 30, 60, and 180 days after application (DAA), either for App.#1 or App.#2. 50 mg sub-samples of fresh leaf tissue were allocated in Eppendorf tubes with 1.5 mL of HCl 0.25 N, frozen in liquid nitrogen, and stored at -40 °C for further analysis. The Eppendorf tubes were defrosted at room temperature and then incubated for 45 min at 37 °C. Aliquots of 50 µL were transferred to new Eppendorf tubes containing 200 µL of periodic acid 0.25% (w/v) and sodium meta periodate 0.25% (w/v). The samples were incubated one more time at 37 °C for 30 min, and then 200 µL of sodium hydroxide 0.6 N + sodium sulfite 0.22 N were added and homogenized. Volumes of 300 µL were transferred to spectrophotometric cuvettes containing 600 µL of distilled water. Absorbance was measured at a wavelength of 380 nm in a diode array spectrophotometer (HP 8425A, Palo Alto, CA, USA). *Shk* concentration was expressed as µmol g<sup>-1</sup> of fresh tissue.

## 2.3. Evaluation of CO<sub>2</sub> assimilation by the chlorophyll- $\alpha$ fluorescence and gas exchanges

Gas exchange and fluorescence of chlorophyll- $\alpha$  were measured on the exact dates as 2.2 through a gas exchange analyzer integrated with a fluorescence chamber and CO<sub>2</sub> analyzer by infrared radiation and light source (IRGA, Li-6800 coupled with fluorescence chamber, Li-Cor Inc., Lincoln, USA). Measurements were taken during the morning (between 8 and 9 h) in median maturation leaves of the mid-third of the plant (sink leaves), fully exposed to light. The following parameters were set: flow density of photosynthetically active photons (800 µmol m<sup>-2</sup> s<sup>-1</sup>), 400 µmol CO<sub>2</sub> mol<sup>-1</sup>, relative humidity of the chamber (50%), airflow (500 µmol s<sup>-1</sup>), and air temperature (30 °C).

For chlorophyll- $\alpha$  fluorescence in light-adapted leaves, the measured variables were: quantum productivity based on electron transport through the photosystem II ( $\Phi_{PSII}$ ), quantum productivity based on CO<sub>2</sub> assimilation ( $\Phi_{CO_2}$ ), apparent electron transport (*ETR*, µmol electrons m<sup>-2</sup>s<sup>-1</sup>), the quantum efficiency of photosystem II (PSII) ( $F_v'/F_m'$ ), photochemical quenching (by the proportion of open PSII reaction centers) (*qP*), and non-photochemical quenching (*NPQ*). Regarding gas exchange, the measured variables were: net CO<sub>2</sub> assimilation rate ( $A_N$ , µmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup>), stomatal conductance to water vapor ( $g_s$ , mol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>), transpiration rate ( $E$ , mmol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>), the intercellular concentration of CO<sub>2</sub> ( $C_i$ , µmol CO<sub>2</sub> mol<sup>-1</sup>), carboxylation efficiency ( $A_N/C_i$ ), the efficiency of water use ( $A_N/E$ ), and intrinsic efficiency of water use ( $A_N/g_s$ ).

## 2.4. Visual symptoms of glyphosate phytotoxicity on citrus plants

Leaf visual symptoms were evaluated at 30, 60, and 180 DAA for both applications. The plants were observed regarding the incidence of the most common symptoms associated with glyphosate phytotoxicity symptoms (adapted from Martinelli et al., 2022): mild chlorosis (up to 10% of leaves), intermediate (10–30% of leaves), and high intensity (>30% of leaves), mild defoliation (up to 10% leaf drop), intermediate (10–30% leaf drop), and high intensity (>30% leaf drop), leaf anatomical deformation (which become more lanceolate) at mild (up to 10% of leaves), intermediate (10–30% of leaves), and high intensity (>30% of leaves), sprouts presence or absence, excessive budding (abnormal branches with increased shoots) with apparent shortening of internodes and an increase in the number of branches and leaves, and stunting. A binomial system for evaluating the presence (1) or absence (0) of the characteristic symptoms was used.

## 2.5. Fruit production

The production of Valencia orange plants was evaluated at 60 DAA#2 (January/2021) in the primary ripening season for this variety. The fruits were harvested and weighed for each plant, and the production data was presented relative to the control.

## 2.6. Data analysis

The data presented normality and were submitted to analysis of variance (ANOVA), and Tukey's HSD (Honestly Significant Difference;  $\alpha = 0.05$ ) was used when necessary. Correlations between variables were submitted by Pearson's linear correlation test ( $\alpha = 0.05$ ). Linear regressions were used for  $\Phi_{PSII}$  and *ETR*. Non-linear regressions were used for  $A_N/g_s$ ,  $A_N/E$ , and  $A_N/C_i$ , as the used exponential model was:  $y = y_0 + Ae^{-\frac{R}{R_0}x}$ , where  $y_0$  corresponds to the asymptote value of the curve,  $A$  to the initial value, and  $R_0$  to the rate of decrease. For the visual phytotoxicity symptoms (non-parametric qualitative data), Pearson's Chi-square test ( $\chi^2$ ;  $p < 0.05$ ) was used within the treatments. Normality tests, Pearson's correlations, and ANOVA were performed in the R software (v 4.1.0) (R Development Core Team 2021), using the *agricolae* package for means separation tests (De Mendiburu, 2021), and the *drc* package for non-linear regressions (Ritz and Streibig, 2021).

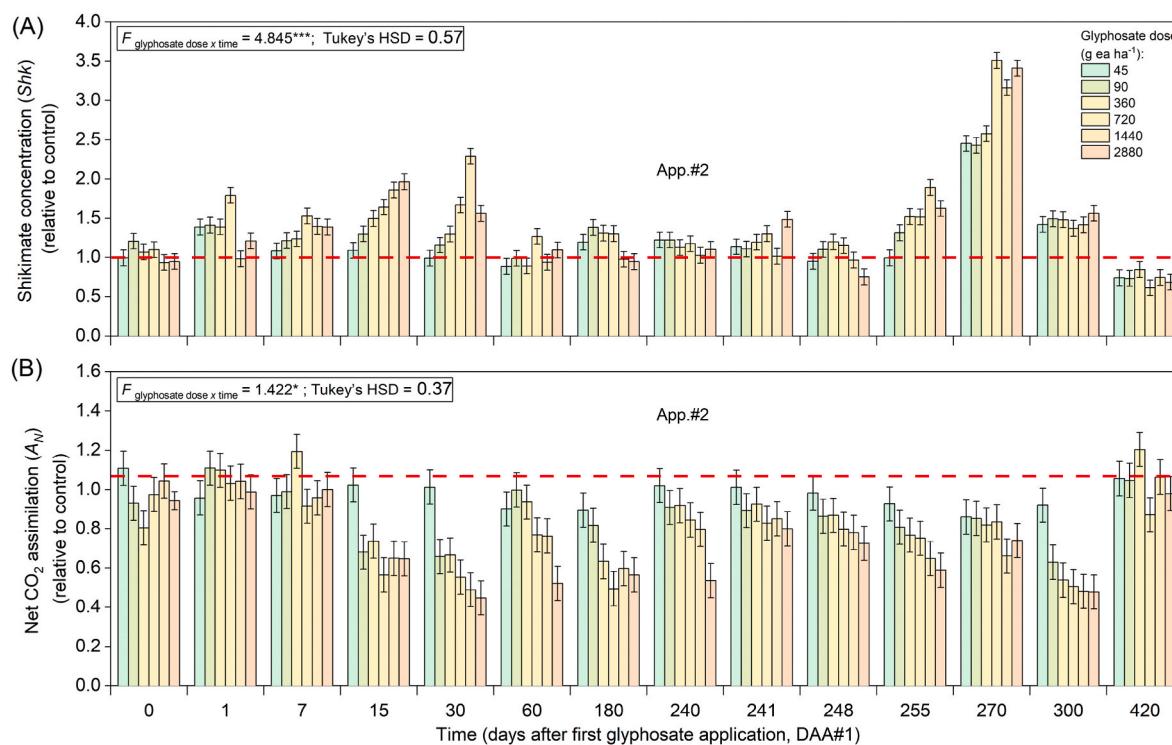
## 3. Results

### 3.1. Shikimate accumulation and net photosynthesis

*Shk* accumulation and net CO<sub>2</sub> assimilation varied as a function of the glyphosate dose over time ( $p$  glyphosate doses x time <0.001). *Shk* accumulation was occasionally related to a gradual glyphosate dose-response (Fig. 1A). On the first day after the first application (1 DAA#1), *Shk* accumulation at 720 g ae ha<sup>-1</sup> dose was 1.8 times higher than the control (0 g ae ha<sup>-1</sup>) ( $p = 0.001$ ). At 15 DAA#1, *Shk* accumulation was a dose-response, proportionally increasing from 720 g ae ha<sup>-1</sup> onwards, reaching ~2.0 times the control for the highest dose (2880 g ae ha<sup>-1</sup>) ( $p$  720 g ae ha = 0.018;  $p$  1440 g ae ha <0.001;  $p$  2880 g ae ha <0.001). At 30 DAA#1, the highest *Shk* accumulations, of ~1.7 and ~2.3 times the control, were respectively recorded at 720 g ae ha<sup>-1</sup> ( $p = 0.012$ ) and 1440 g ae ha<sup>-1</sup> ( $p < 0.001$ ). At 60 DAA#1, *Shk* concentration decreased significantly with values similar to the control until the second glyphosate application (240 DAA#1 = 0 DAA#2) ( $p > 0.05$ ).

At 15 DAA#2, *Shk* accumulations were ~1.6 and ~1.9 times higher than control, respectively, at 1440 g ae ha<sup>-1</sup> ( $p < 0.001$ ) and 2880 g ae ha<sup>-1</sup> ( $p = 0.024$ ) (Fig. 1A). At 30 DAA#2, the highest *Shk* concentrations of the entire experiment were observed from 720 g ae ha<sup>-1</sup> dose onwards, being ~3.2 to ~3.5 times higher than control ( $p < 0.001$ ). Likewise, treatments that did not show differences for App.#1, such as 90 and 360 g ae ha<sup>-1</sup>, accumulated *Shk* for the first time (~2.5 times higher than the control;  $p < 0.001$ ). From 60 DAA#2, *Shk* concentration decreased to the control levels until the end of the experiment (180 DAA#2 = 420 DAA#1). It is noteworthy that there was a cumulative effect with the consecutive glyphosate application, as *Shk* concentration peaks for each dose in App.#2 were ~2.1, ~1.7, ~2.0, ~1.4, and ~1.7 times higher than the peaks from App.#1, respectively for 360 (at 15 DAA#1), 720 (at 1 DAA#1), 1440 (at 30 DAA#1), and 2880 g ae ha<sup>-1</sup> (at 15 DAA#1) ( $p < 0.05$ ).

For  $A_N$ , at 15 DAA#1, there was a 44% decrease relative to the control at 720 g ae ha<sup>-1</sup> ( $p = 0.071$ ) (Fig. 1B), which remained until 30 DAA#1 ( $p = 0.005$ ). On the same date, the doses of 1440 and 2880 g ae ha<sup>-1</sup> also decreased ~55% ( $p < 0.001$ ). This was also observed at 180 DAA#1 for 1440 g ae ha<sup>-1</sup> ( $p = 0.017$ ) and 2880 g ae ha<sup>-1</sup> ( $p = 0.007$ ), which remained until the second glyphosate application (240 DAA#1) ( $p < 0.05$ ). At 15 DAA#2, the 2880 g ae ha<sup>-1</sup> showed a 41% reduction ( $p = 0.014$ ), and at 60 DAA#2, all doses from 360 g ae<sup>-1</sup> onwards reduced  $A_N$  by ~56% ( $p < 0.01$ ).



**Fig. 1.** Glyphosate doses ( $\text{g ae ha}^{-1}$ ) and time (days after first glyphosate application, DAA#1) for (A) shikimate concentration (*Shk*) (relative to control), and (B) the net  $\text{CO}_2$  assimilation ( $A_N$ ) (relative to control). Glyphosate's second application (App.#2) date and Tukey's HSD ( $\alpha = 0.05$ ) values for comparison within glyphosate doses and times are indicated. Error bars represent the standard error of the mean ( $\pm$ ). The red dashed line refers to the control treatment values.  $n = 7$  treatments  $\times$  3 replications  $\times$  14 evaluated dates = 294. Control treatment absolute values respectively for 0, 1, 7, 15, 30, 60, 180, 240 241, 247, 255, 270, 300 and 420 DAA#1 were: for *Shk*, 12.5, 16.6, 15.3, 15.6, 13.7, 21.5 19.6, 24.8, 20.0, 20.5, 7.9, 5.7, 15.8, and 11.4  $\mu\text{mol g}^{-1}$  of fresh tissue; and, for  $A_N$ , 6.3, 7.1, 6.4, 7.9, 7.6, 6.0, 5.9, 5.6, 7.0, 7.0, 6.8, 8.0, 6.5, and 3.9  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$  respectively for. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Furthermore, inversely proportional relationships between *Shk* and  $A_N$  were observed several dates after each glyphosate application, at 15 and 30 DAA#1 and 15, 30, and 60 DAA#2 (Table 1).

### 3.2. Leaf chlorophyll- $\alpha$ fluorescence

$\Phi_{\text{PSII}}$  and ETR varied only as a function of the glyphosate doses ( $p < 0.001$ ), i.e., there was no time effect ( $p_{\text{glyphosate doses} \times \text{time}} > 0.05$ ).  $\Phi_{\text{PSII}}$  decreased linearly with the increase of glyphosate dose (Fig. 2A), causing an ETR reduction of  $\sim 3.7 \mu\text{mol electrons m}^{-2} \text{ s}^{-1}$  for each increase of  $1000 \text{ g ae ha}^{-1}$  of glyphosate ( $p < 0.001$ ), which corresponds to a 17% drop during the entire experimental period.

$\Phi_{\text{CO}_2}$ ,  $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ ,  $F_v'/F_m'$  and NPQ varied as a function of the glyphosate dose over time ( $p_{\text{glyphosate doses} \times \text{time}} < 0.001$ ), with main differences at two specific dates each. At 1 DAA#1,  $\Phi_{\text{CO}_2}$  decreased up to 2.0 times compared to the control for the lowest ( $p_{45 \text{ g ae ha}^{-1}} = 0.001$ ) and highest glyphosate doses ( $p_{2880 \text{ g ae ha}^{-1}} < 0.001$ ) (Fig. 2C). The same decrease level in  $\Phi_{\text{CO}_2}$  occurred for 45  $\text{g ae ha}^{-1}$  at 30 DAA#2, and consequently, superior decreases (4.1 times) were observed at 720 and 1440  $\text{g ae ha}^{-1}$  ( $p < 0.001$ ). In addition, for  $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ , increases of 2.4 and 3.2 times higher than control were observed for 45 and 90  $\text{g ae ha}^{-1}$  at 15 DAA#1 ( $p < 0.001$ ), respectively. At 30 DAA#2, there was an increase of 6.2 and 3.6 times, respectively for 720  $\text{g ae ha}^{-1}$  ( $p < 0.001$ ) and 1440  $\text{g ae ha}^{-1}$  ( $p = 0.049$ ) (Fig. 2D).

$F_v'/F_m'$  and NPQ were affected by glyphosate on the same dates (Fig. 2E and F).  $F_v'/F_m'$  decreased 42% relative to the control at 240 DAA#1 at 720  $\text{g ae ha}^{-1}$  ( $p < 0.001$ ), while at 180 DAA#2, there was a 30% decrease at 720  $\text{g ae ha}^{-1}$  compared to control ( $p = 0.007$ ) and 2880  $\text{g ae ha}^{-1}$  ( $p = 0.024$ ) (Fig. 3E). Furthermore, proportional relationships between  $\Phi_{\text{PSII}}$  and  $F_v'/F_m'$  were observed in half of the evaluated dates (Table 1). The NPQ presented two peaks at 240 DAA#1, and

first with a decrease by half relative to the control at 360  $\text{g ae ha}^{-1}$  ( $p = 0.030$ ), secondly with an increase of 2.6 times at 360–720  $\text{g ae ha}^{-1}$  ( $p < 0.001$ ), and with another decrease of 2.5 times at 720–2880  $\text{g ae ha}^{-1}$  ( $p < 0.001$ ) (Fig. 2F). At 180 DAA#2, the plants showed similar behavior, with 2.8 times increase in NPQ at 90–720  $\text{g ae ha}^{-1}$  ( $p = 0.026$ ) and 2.3 times decrease at 720–2880  $\text{g ae ha}^{-1}$  ( $p = 0.041$ ).

Inversely proportional relationships of NPQ and  $F_v'/F_m'$  were observed in 10 of the 14 dates evaluated, showing that the decrease in  $F_v'/F_m'$  increased the demand for NPQ (Table 1). The same occurred between  $qP$  with  $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$ . However, these two variables presented a proportional relationship before the first glyphosate application, as the relationship became inversely proportional after the applications, with decreases in  $qP$  (Fig. 2C). Thus, proportional relationships between  $qP$  and  $\Phi_{\text{CO}_2}$  were observed in more than half of the evaluated dates. For photochemical quenching ( $qP$ ), no direct influence of the herbicide was observed ( $p > 0.05$ ), presenting an average value of 0.387 for the entire experimental period.

### 3.3. Leaf gas exchange

Leaf gas exchange variables varied only as a function of glyphosate dose ( $g_s$ ,  $p = 0.011$ ;  $C_i$ ,  $p < 0.001$ ;  $E$ ,  $p < 0.001$ ;  $A_N/g_s$ ,  $p < 0.020$ ;  $A_N/C_i$ ,  $p < 0.001$ ;  $A_N/E$ ,  $p < 0.001$ ).  $g_s$  decreased 20% only at 360  $\text{g ae ha}^{-1}$  (Fig. 3A) ( $p < 0.001$ ); however,  $A_N/g_s$  presented a negative exponential response with the increase of glyphosate, with a decrease between 8 and 26% from 360 to 2880  $\text{g ae ha}^{-1}$  (Fig. 3B). These results show that stomatic resistance (due to the lowest values of  $g_s = -20\%$ ) had little influence on  $A_N/g_s$  (-8%). However, at 360  $\text{g ae ha}^{-1}$  dose onwards, the  $A_N/g_s$  decreased exponentially, even with the inversion of  $g_s$  values (+20%).

$E$  and  $A_N/E$  presented similar patterns to the  $g_s$ , as  $C_i$  and  $A_N/C_i$  to

**Table 1** Pearson's correlations between variables over time. Quantum efficiency of photosystem II (PSII) ( $F_v/F_m'$ ); quantum efficiency of electron transport through photosystem II (PSII) ( $\Phi_{PSII}$ ); quantum productivity based on CO<sub>2</sub> assimilation ( $\phi_{CO_2}$ ); non-photochemical quenching ( $qP$ ); photochemical quenching ( $NPQ$ ); stomatal conductance ( $g_s$ ; mol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>); intercellular CO<sub>2</sub> concentration ( $Ci$ ; µmol CO<sub>2</sub> mol<sup>-1</sup>); transpiration rate ( $E$ ; mmol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>); efficiency of carboxylation ( $A_N/Ci$ ); concentration of shikimate ( $Shk$ ); net assimilation of CO<sub>2</sub> ( $A_N$ ).

DAA	$A_N$		$Fv'/Fm'$		$qP$		$g_s$		$A_N/Ci$	
	$v. Shk$	$v. A_N/Ci$	$v. \Phi_{PSII}$	$v. NPQ$	$v. \phi_{CO_2}$	$v. \phi_{CO_2}$	$v. Ci$	$v. E$	$v. Shk$	$v. \Phi_{PSII}$
0 (App.#1)	ns	0.79 ***	0.45 *	-0.61	**	0.58	**	0.64	**	0.99 ***
1	ns	0.85 ***	ns	-0.56	**	ns	0.70	**	0.61	**
7	ns	0.89 ***	ns	-0.70	***	ns	0.79	**	0.81	***
15	-0.74 ***	0.88 ***	ns	-0.88	***	-0.51	*	0.56	**	ns
30	-0.69 ***	0.96 ***	0.55 **	ns	**	ns	0.71	***	0.74	**
60	ns	0.83 ***	ns	-0.52	*	-0.45	*	0.63	**	ns
180	ns	0.93 ***	ns	-0.66	**	-0.58	**	0.53	*	ns
240 (App.#2)	ns	0.94 ***	0.54 *	* -0.71	***	ns	ns	ns	0.99 ***	ns
241	ns	0.94 ***	0.50 *	ns	**	ns	ns	ns	ns	0.63 ***
248	ns	0.97 ***	0.63 **	ns	**	ns	0.49	*	ns	0.73 ***
255	-0.46 *	0.95 ***	0.44 *	ns	**	ns	0.49	*	0.52	*
270	-0.47 *	0.54 *	0.66 **	ns	-0.52	*	ns	0.66 **	ns	-0.57 *
300	-0.50 *	0.94 ***	ns	-0.79 ***	-0.50	*	0.76	***	ns	-0.47 *
420	ns	0.85 ***	ns	-0.93 ***	ns	ns	0.54	*	ns	0.55 **

DAA: days after application; App.#1 and App.#2: glyphosate applications; p values, \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ ; ns: not significant ( $p > 0.05$ ).

$A_N/g_s$ .  $E$  increased 63% as  $A_N/E$  decreased 40% both at 1440 g ae ha<sup>-1</sup> ( $p < 0.001$ ), which was very close to the maximum observed decrease (43% at ~2200 g ae ha<sup>-1</sup>) ( $p < 0.001$ ) (Fig. 3C and D).  $Ci$  decreased by 8% at 360 g ae ha<sup>-1</sup> ( $p = 0.013$ ), as  $A_N/Ci$  decreased by 12% also at 360 g ae ha<sup>-1</sup>, with a maximum decrease for  $A_N/Ci$  of 27% decrease at ~2700 g ae ha<sup>-1</sup> ( $p < 0.001$ ) (Fig. 3E and F).

Notably, inversely proportional relationships between  $Shk$  and  $A_N/Ci$  were observed several dates after each glyphosate application, indicating that the  $Shk$  accumulation decreased  $A_N/Ci$  levels (Table 1).

### 3.4. Phytotoxicity levels and symptoms incidence

Only chlorosis, defoliation, and leaves anatomical deformation were observed (Figs. 4 and 5). Mild chlorosis symptoms on citrus leaves were observed from 30 DAA#1, without statistical differences between glyphosate doses [ $X^2$  (6, N = 21) = 57.7;  $p < 0.001$ ] (Figs. 4A and 5B). Although, at 180 DAA#1, an increase of this symptom on 1440 and 2880 g ea ha<sup>-1</sup> was detected, which became more frequent after App. #2, as 100% of the plants showed this symptom from 720 g ea ha<sup>-1</sup> onwards. Intermediate intensities of chlorosis were only presented at 180 DAA#2 for 100% of the plants from 720 g ea ha<sup>-1</sup> onwards [ $X^2$  (6, N = 21) = 22.7;  $p < 0.001$ ] (Fig. 4B). Persistent defoliation at mild intensity was presented from 30 DAA#1, ranging from 67% to 100% of the plants with doses from 720 g ea ha<sup>-1</sup> onwards, which remained until 180 DAA#2 at ≥67%, from 45 g ea ha<sup>-1</sup> [ $X^2$  (6, N = 21) = 71.3;  $p < 0.001$ ] (Fig. 4C). Intermediate defoliation level was observed at 180 DAA#1 by 67% of the plants for 2880 g ea ha<sup>-1</sup>, and at 180 DAA#2 with 100% of the plants from 720 g ea ha<sup>-1</sup> onwards [ $X^2$  (6, N = 21) = 38.3;  $p < 0.001$ ] (Figs. 4D and 5C). Continuous leaf deformation at mild intensity was observed at 60 DAA#1 until the end of the experiment, ranging from 33% to 67% of the plants at 720 g ea ha<sup>-1</sup>, 67%–100% at 1440 g ea ha<sup>-1</sup>, and 100% at 2880 g ea ha<sup>-1</sup> [ $X^2$  (6, N = 21) = 80.7;  $p < 0.001$ ] (Figs. 4E and 5D-F).

### 3.5. Fruit production from the glyphosate effect

All the effects of glyphosate on citrus plants observed in this work contributed to a lower fruit yield with the increased glyphosate dose and the  $Shk$  accumulation at 60 DAA#2 (Fig. 6). Fruit production losses were observed as negative exponential results, with decreases of 30% at 1000 g ae ha<sup>-1</sup>, 47% at 2000 g ae ha<sup>-1</sup>, reaching decreases of up to 57% at 2880 g ae ha<sup>-1</sup> ( $p < 0.001$ ). Furthermore, inversely proportional relationships between fruit production with  $Shk$  ( $p = 0.020$ ) and  $A_N$  ( $p = 0.003$ ) were detected (Fig. 6B and C).

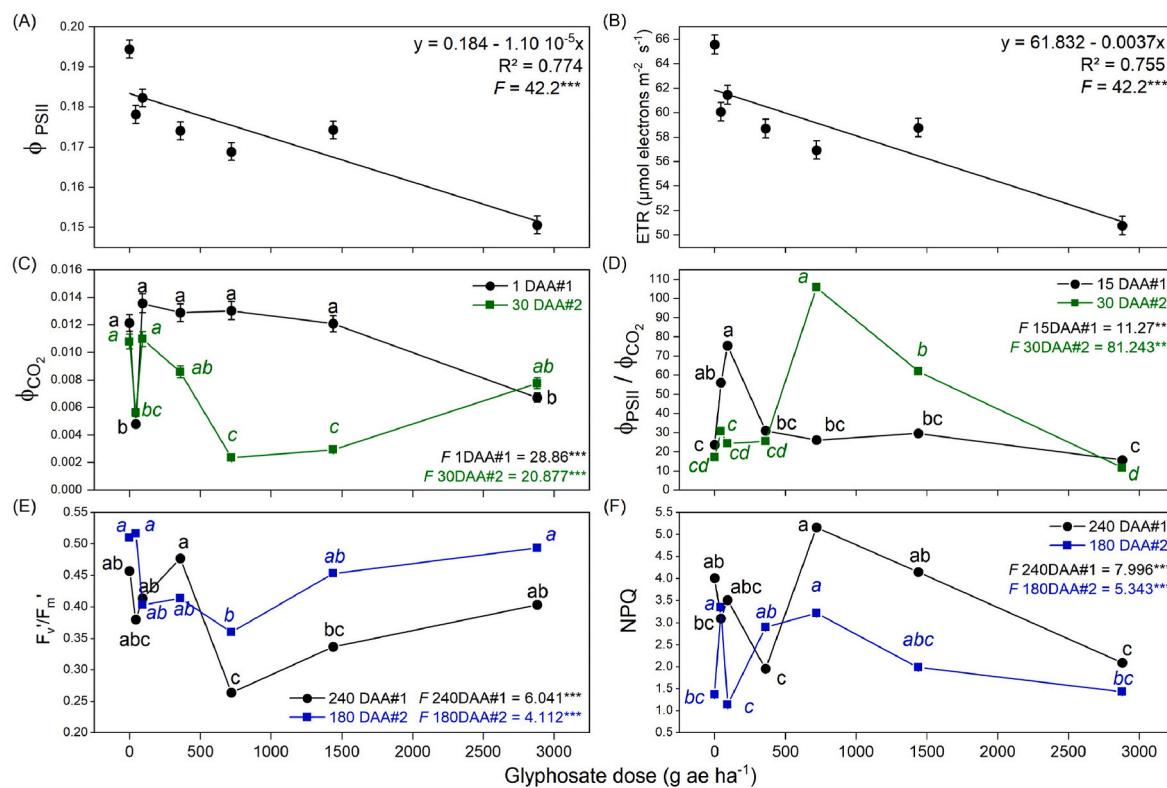
A schematic representation of the main effects detected in this study is presented (Fig. 7).

## 4. Discussion

### 4.1. Shikimate accumulation

$Shk$  accumulation is a biochemical indicator widely used to evaluate the effects of glyphosate on plants (Singh and Shaner, 1998). Untreated sink leaves (middle third) of citrus plants accumulated  $Shk$  by glyphosate applications on source leaves (lower third). This methodology was adapted to simulate field conditions concerning herbicide drift, but, in addition, it also showed the high translocation capacity of glyphosate in adult citrus plants (with lignified shoots) and its impacts on photosynthesis from the first days after App.#1. Historically glyphosate has been widely used for weed control in perennial tree crops considering that the absorption and translocation of this herbicide are limited in lignified tissues (Chen et al., 2009). However, recent studies have shown that glyphosate can be absorbed and transferred in shrub and arboreal plants at rates similar to those observed in herbaceous plants (Kogan and Alister, 2010; Tong et al., 2017).

Three previous studies reported that citrus plants are tolerant to



**Fig. 2.** Linear regressions and one-way ANOVA for chlorophyll fluorescence variables under glyphosate doses ( $g\text{ ae ha}^{-1}$ ): (A) quantum efficiency of electron transport through photosystem II ( $\phi_{PSII}$ ); (B) apparent electron transport (ETR,  $\mu\text{mol electrons m}^{-2} \text{s}^{-1}$ ); (C) quantum productivity based on  $\text{CO}_2$  assimilation ( $\phi_{CO_2}$ ); (D) ratio between  $\phi_{PSII}$  and  $\phi_{CO_2}$ ; (E) quantum efficiency of photosystem II ( $F_v'/F_m'$ ); (F) non-photochemical quenching (NPQ). Error bars represent the standard error of the mean ( $\pm$ ). A-B figures,  $n = 7$  treatments  $\times$  3 replications  $\times$  14 times = 294. C-F figures,  $n = 42$ . The 240 DAA#1 (days after the first application) date also refers to 0 DAA#2. For C-F figures, means followed by the same letter within each interaction do not differ by Tukey's HSD ( $\alpha = 0.05$ ).  $p$  values: \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ .

glyphosate drift and that plants do not accumulate Shk. Rangpur lime seedlings did not accumulate Shk with doses up to  $720\text{ g ae ha}^{-1}$ , although Shk was quantified only up to eight DAA (Gravena et al., 2009). Two applications of 1440 and  $1260\text{ g ae ha}^{-1}$ , carried out with a tractor and anti-drift accessories at an interval of 3.5 months, did not induce Shk accumulation in a 12-yr-old Péra sweet orange orchard up to 35 DAA (Matallo et al., 2010). In a young Valencia sweet orange orchard with two rootstocks (Rangpur lime and citrumelo Swingle [*Poncirus trifoliata* (L.) Raf. x *C. paradisi* Macf.]), simulated glyphosate drifts were made on the trunk (doses up to  $2160\text{ g ae ha}^{-1}$ ) or in predominantly green shoots (doses up to  $720\text{ g ae ha}^{-1}$ ) (Gravena et al., 2012). However, no Shk accumulation was found at 8 and 15 DAA; treated plants only showed visual phyto intoxication symptoms and smaller trunk diameters.

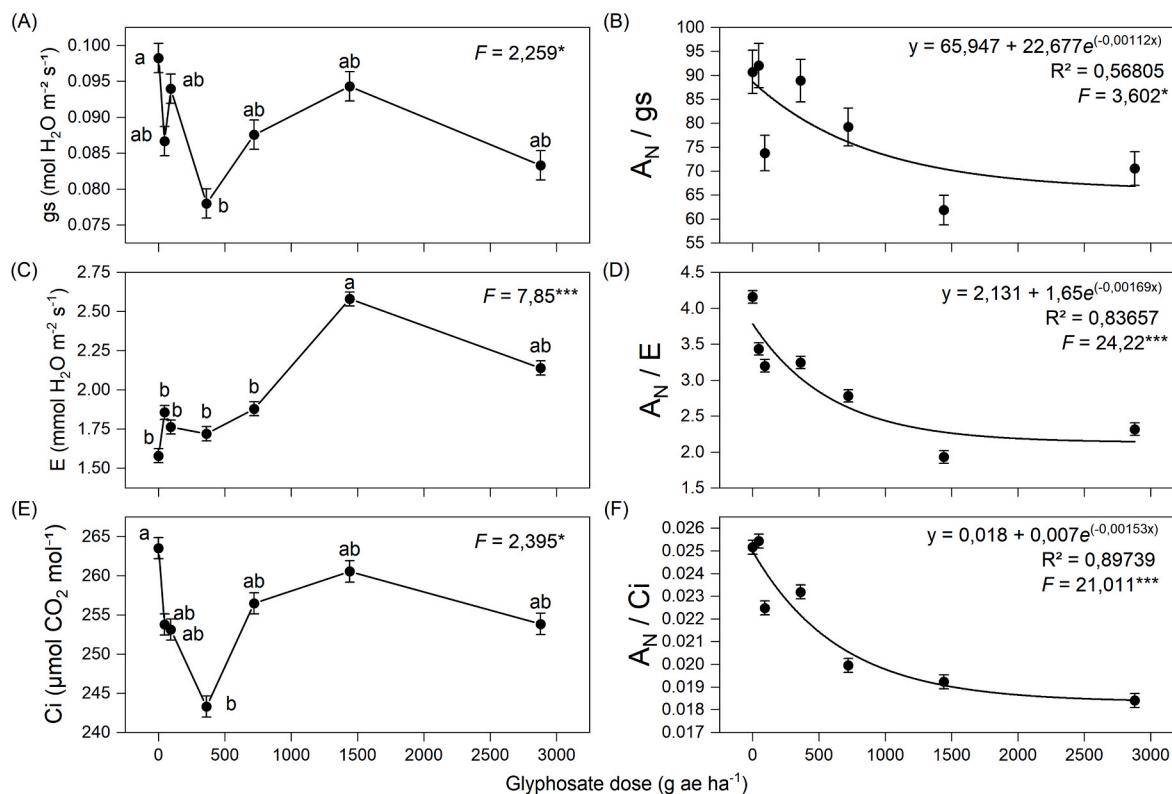
Our results contrast these studies, as Shk accumulation was rapidly detected just one day after the first glyphosate application. The most significant differences were detected up to 30 days after both applications. Also, the accumulation peaked at the second application with even higher accumulation values, which corroborates that Shk can be used as a bioindicator of glyphosate phyto intoxication for citrus crops. Furthermore, this is the first study to report on the long-term accumulation of Shk in citrus plants.

#### 4.2. Glyphosate and shikimate accumulation affect citrus photosynthesis

Various parameters of photosynthesis, such as gas exchange and chlorophyll- $\alpha$  fluorescence, were impacted in citrus plants by glyphosate-induced Shk accumulation, as corroborated by the correlations between them on several evaluated dates (Table 1). For the leaf chlorophyll- $\alpha$  fluorescence, the first observed effect was a drop in  $\phi_{PSII}$  and ETR over a prolonged period (420 d).  $\phi_{PSII}$  can provide a measure of

the linear ETR and photosynthetic capacity *in vivo* (Genty et al., 1989), suggesting that glyphosate damage may promote a greater demand for luminous energy (photons) to be absorbed by chlorophylls (associated with PSII) to drive photochemistry, *i.e.*, to transfer the electrons ( $e^-$ ) from the reaction center chlorophyll (P680) to the primary quinone acceptor of PSII (Q<sub>A</sub>) (Baker, 2008). So, the decreases imposed on  $\phi_{PSII}$  and  $\phi_{CO_2}$  by glyphosate show that the absorption of light exceeded the assimilation requirements of each  $\text{CO}_2$  molecule and that the dissipation of excess energy was ineffective by the first suppression mechanisms (quenching) (Gallé and Flexas, 2010). These results corroborate the importance of evaluating the  $\phi_{PSII}/\phi_{CO_2}$  ratio, since the observed increases suggest that most of the energy absorbed by the photosynthetic apparatus was not channeled to the assimilation of  $\text{CO}_2$ , indicating the existence of non-cyclic  $e^-$  that cause photooxidative damage and an increase in photorespiration (Guo et al., 2006; Baker, 2008). Generally, increases in  $\phi_{PSII}/\phi_{CO_2}$  are accompanied by increases in the activity of antioxidant enzymes involved in the elimination of reactive oxygen species (ROS), suggesting that there was an increase in the flow of  $e^-$  to  $O_2$  rather than to  $\text{CO}_2$  assimilation through the Mehler reaction, reducing  $O_2$  to superoxide anion  $O_2^-$  by donation of an  $e^-$  (Baker, 2008). These effects were demonstrated in a study with citrus plants under thermal stress since  $A_N$  and  $\phi_{CO_2}$  were more affected by the increase in photorespiration/ $A_N$  and  $\phi_{PSII}/\phi_{CO_2}$  ratios (Guo et al., 2006).

The  $F_v'/F_m'$  of citrus plants decreased following the  $\phi_{PSII}$  decreases, suggesting that glyphosate promotes a fast PSII saturation, as demonstrated by the integrity loss that can decrease ATP and NADPH production (Zobiole et al., 2010; Gomes et al., 2017). These results were corroborated by the increase in NPQ due to a demand increase for quenching processes. Increasing NPQ and  $qP$  are the primary processes involved in photoprotection, intentionally reducing  $\phi_{PSII}$  to maintain a



**Fig. 3.** One-way ANOVA and exponential regressions for gas exchange variables under the different doses of glyphosate (g ae ha<sup>-1</sup>): (A) stomatic conductance ( $g_s$ ); (B) intercellular  $\text{CO}_2$  concentration ( $C_i$ ); (C) transpiration rate ( $E$ ); (D) efficiency of water use ( $A_N/E$ ); (E) intrinsic efficiency of water use ( $A_N/gs$ ); (F) efficiency of carboxylation ( $A_N/C_i$ ). Error bars represent the standard error of the mean ( $\pm$ ). Because there were no interactions between glyphosate dose and time, the variables represent the mean values of each treatment of all the evaluations ( $n = 7$  treatments  $\times$  3 replications  $\times$  14 evaluated times = 294). For figures A, C, and E, means followed by the same letter within each interaction do not differ by Tukey's HSD ( $\alpha = 0.05$ ).  $p$  values: \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ .

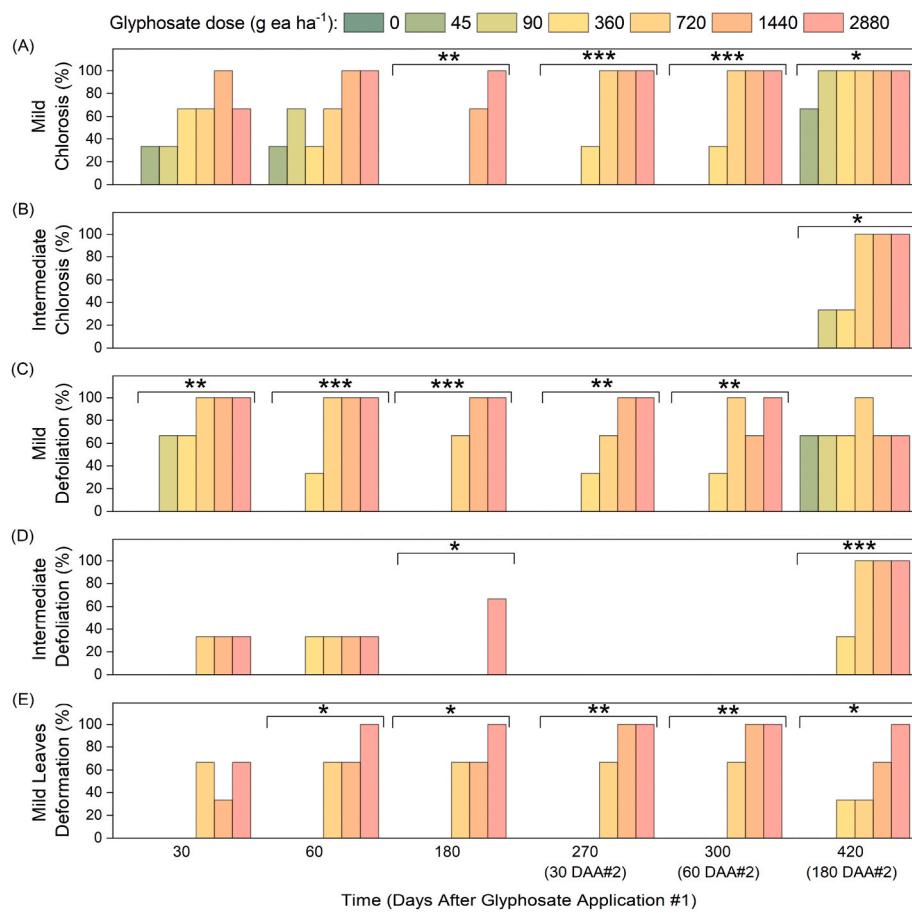
balance with the  $e^-$  flow demand for C metabolism (Gallé and Flexas, 2010). In willow (*Salix miyabeana* Seemen) treated with glyphosate, a well known phytoremediation plant that is highly tolerant to stresses, first, there was *Shk* accumulation, and consequently, a decrease in *ETR*, *qP*, and maximum photochemical efficiency of PSII ( $F_v/F_m$ ; on dark-adapted leaves) (Gomes et al., 2017). These alterations reduced the content of chlorophyll, carotenoid, and plastoquinone, resulting in the accumulation of hydrogen peroxide ( $H_2\text{O}_2$ ) and proline due to the low (decreased) activity of antioxidant enzymes (Gomes et al., 2017; Taiz et al., 2017). ROS production may have caused the reduction in chlorophyll fluorescence due to the excessive excitation of chlorophylls: a result of the decreases in  $\Phi_{\text{PSII}}$ , *ETR*, and  $F_v'/F_m'$ , and increases in *NPQ*, as observed in this study; indirectly, *qP* decreased by its relationship with  $\Phi_{\text{PSII}}/\Phi_{\text{CO}_2}$  and  $\Phi_{\text{CO}_2}$  (Table 1).

Regarding gas exchange, glyphosate ( $>360$  g ae ha<sup>-1</sup>) decreased stomatal resistance ( $g_s$  increase), increasing the  $\text{CO}_2$  influx ( $C_i$  increase); however, with substantial water loss ( $E$  increase) and proportional relationships on several dates (Table 1). In other words, glyphosate prevented citrus plants from assimilating  $\text{CO}_2$ , even with a higher  $\text{CO}_2$  influx, which may be related to water loss (by higher  $E$  values) and maintaining the values of  $g_s$  and  $C_i$ , resulting in higher levels of  $A_N/gs$  and  $A_N/C_i$ , and  $A_N/E$ . This significant water loss affected all processes dependent on  $e^-$  transfer, directly affecting  $A_N$  since these are derived from water oxidation and the processes that require ATP and NADPH (Taiz et al., 2017). It is known that in optimal conditions,  $C_i$  and  $g_s$  have an inversely proportional relationship in citrus plants (without glyphosate application), where  $C_i$  drops stimulate the opening of stomata to promote  $\text{CO}_2$  influx into the substomatal cavity and keep  $C_i$  levels constant (Machado et al., 2005). Under abiotic stress (elevated temperature and solar radiation), citrus plants presented the same decrease in  $C_i$  and  $g_s$  compared to plants without stress; however, without

differences in  $A_N$  (Boaretto et al., 2020). In a previously mentioned study, glyphosate-treated citrus plants presented constant levels of  $C_i$ , but there were no changes in  $\text{CO}_2$  assimilation rates at 8 DAA; however,  $g_s$  and  $E$  decreased with increasing doses (Gravena et al., 2009).

Glyphosate has contrasting relationships with leaf gas exchanges between distinct species. In a study with *Abutilon theophrasti*, source leaves treated with glyphosate showed decreases in  $A_N$  and  $g_s$  at 2 h after its application (HAT), which was reflected in  $C_i$  decrease (7–19 HAT) and  $A_N/E$  increase (19 HAT) (Fuchs et al., 2002). The same trend was observed one day before the leaves completely lost their function (5 DAA). Increased stomatal resistance rapidly inhibited photosynthetic processes, allowing C fixation to deplete  $C_i$  faster than it could be replaced. In the same study, *Beta vulgaris* L. presented a compensatory reduction of  $g_s$  in response to the biochemical blockade of the Calvin-Benson cycle (CB cycle) by glyphosate, keeping  $C_i$  constant and inhibiting more  $A_N$  than  $E$ . Therefore, for *A. theophrasti*, glyphosate inhibited biochemical processes more than gas exchange processes due to more significant drainage of photosynthesis metabolites (Rubisco and 3-phosphoglycerate). In contrast, for *B. vulgaris* results, the gas exchange processes affected  $\text{CO}_2$  assimilation, similar to the results of the present study.

Most plant leaves regulate  $C_i$  by the stomatic opening, so it remains at intermediate concentrations (and below ambient concentration) between the limits imposed by the carboxylation capacity and the ability to regenerate ribulose-1,5-biphosphate ( $\text{RuP}_2$ ); therefore, stomatic movements provide an opportunity to change the  $E$  and the  $C_i$  at carboxylation sites (Farquhar and Sharkey, 1982). Moreover,  $A_N/C_i$  decreases, even with  $C_i$  increases above 360 g ae ha<sup>-1</sup> (Fig. 3E and F), showing a shift in the fixed  $\text{CO}_2$  balance by the CB cycle, which indicates a partial C drain by the photorespiration pathway; which was corroborated by the inversely proportional relationships of  $A_N/C_i$  and *Shk* (Table 1).



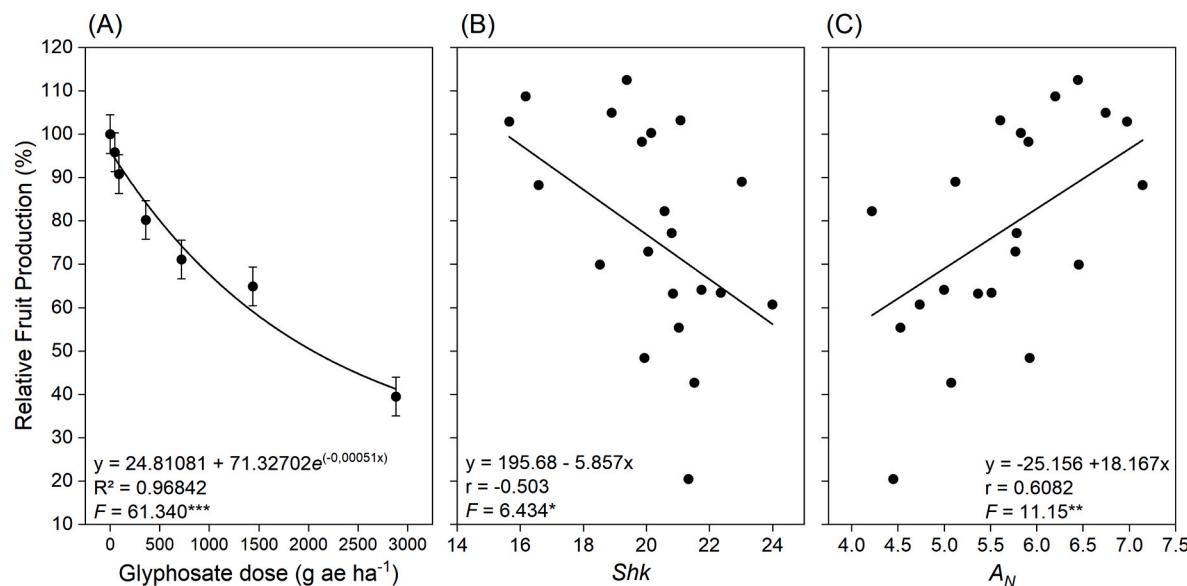
**Fig. 4.** Incidence of the characteristic phytotoxicity symptoms under glyphosate doses ( $\text{g ae ha}^{-1}$ ) at 30, 60, and 180 days after the first glyphosate application (DAA#1 and #2) for Pearson's Chi-square test ( $\chi^2$ ). For chlorosis and leaf deformation results, mild symptoms are classified up to 10% of the leaves, and intermediate symptoms are from 10 to 30%. For defoliation, mild symptoms are classified as up to 10% leaf drop, and intermediate symptoms are from 10 to 30% leaf drop.  $p$  values: \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ .



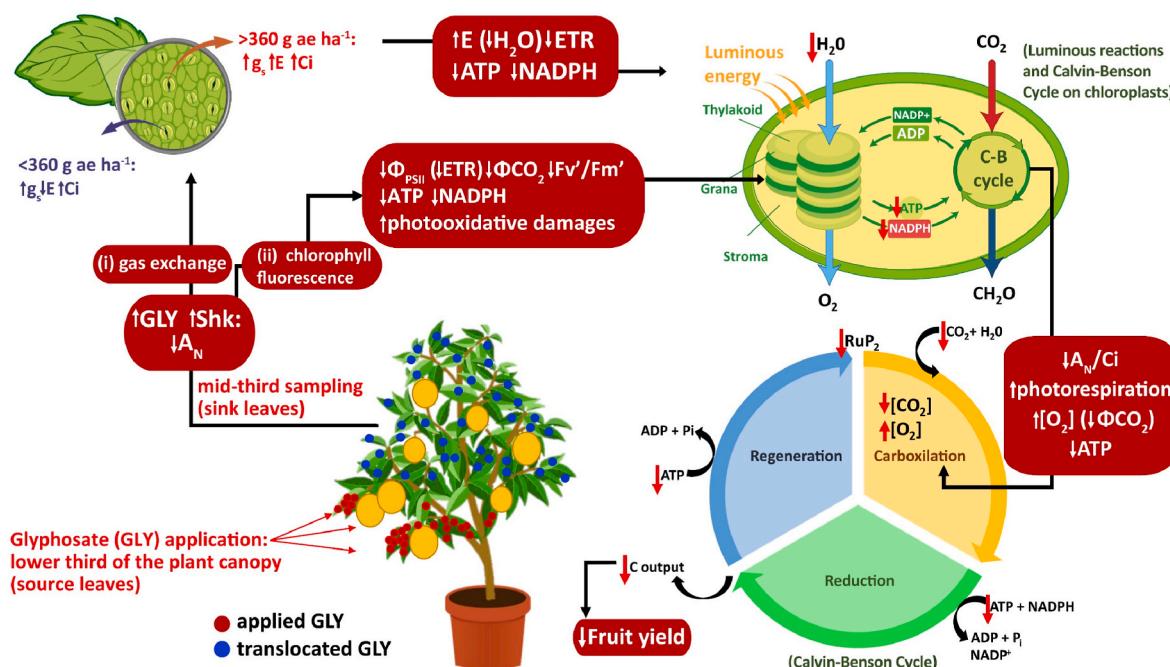
**Fig. 5.** Glyphosate phytoxicity symptoms observed on Valencia orange trees: control treatment – plants without symptoms (A); mild chlorosis (up to 10% of the leaves) (B); intermediate defoliation (10–30% of leaf drop) (C); and leaf deformation (D–F). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In addition, since there was a more significant water loss above 360 g  $\text{ae ha}^{-1}$  (Fig. 3C), high transpiration could also affect the CB cycle's carboxylation phase since a  $\text{CO}_2$ , water, and RuP<sub>2</sub> reaction occur in the first (regeneration) and subsequent CB cycle phases (Taiz et al., 2017).

Another evidence of increased photorespiration in plants treated with glyphosate is the decrease in  $\Phi_{PSII}$  (Fig. 2A), which decreases the production of ATP, NADPH, and RuP<sub>2</sub> for Rubisco (ribulose-1,5-bisphosphate-carboxylase/oxygenase), decreasing the carboxylase activity of



**Fig. 6.** Relative fruit production of the citrus plants (A) and their correlations with the variables: (B) Shikimate concentration ( $Shk$ ); (C) net assimilation rate of  $CO_2$  ( $A_N$ ). All variables are the average values at the fruit harvest date (60 days after application #2). Error bars represent the standard error of the mean ( $\pm$ ).  $p$  values: \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ .



**Fig. 7.** The main glyphosate observed effects on citrus plants in photosynthetic processes.

this enzyme, which consequently reduces the  $Ci$  use (Farquhar and Sharkey, 1982). This increase in photorespiration is corroborated by the proportional relationship detected between  $A_N/Ci$  and  $\Phi_{PSII}$  only on dates close to the glyphosate applications, and mainly after the second application (Table 1).

$A_N$  decreases are expected in  $C_3$  plants (such as citrus) exposed to higher concentrations of  $O_2$  to maximize photorespiration; therefore, they decrease  $\Phi_{CO_2}$  because the quantum demand for  $CO_2$  fixation under photorespiratory conditions (high  $O_2$  and low  $CO_2$ ) is higher than in non-photorespiratory (Farquhar and Sharkey, 1982; Taiz et al., 2017). Reduced carboxylation was highlighted as the main effect correlated with the  $A_N$  decrease caused by glyphosate (Table 1).  $A_N/Ci$  demonstrated proportional relationships on all dates with  $A_N$ , which was

corroborated by the inversely proportional relationships between  $Shk$  and  $A_N/Ci$ , and proportional relationships between  $\Phi_{PSII}$  and  $A_N/Ci$  after glyphosate application. However, this can be a plant strategy to mitigate glyphosate damages, as photorespiration decreases the photosynthesis efficiency by dissipating  $e^-$ , consuming ATP, and NADPH generated by photochemical reactions to protect itself from photoinhibition (Kozaki and Takeba, 1996; Foyer et al., 2009).

Therefore, at the highest doses of glyphosate in citrus plants, it is possible to infer that any protective mechanism was inefficient. First, such as the photoprotective quenchings ( $NPQ$  and  $qP$ ), as already mentioned, and through greater activation of the photorespiratory pathway to dissipate excedent energy. This last demonstrated that it could be a 'high-cost' protective mechanism since a higher activation of

this pathway can act as an additional C drain (Taiz et al., 2017). Also, this is the primary source of H<sub>2</sub>O<sub>2</sub> production in photosynthetic cells, influencing several signaling pathways, such as plant hormonal responses, environmental responses, and plant defense (Foyer et al., 2009).

Also, it is known that the production and metabolism of glyphosate to aminomethylphosphonic acid (AMPA) varies between species, as this is a recognized phytotoxin, and its co-occurrence with glyphosate could affect plant physiology (Gomes et al., 2014; Duke, 2020). Furthermore, it is suggested that AMPA can affect the chlorophyll biosynthetic pathway and the photorespiration process (Gomes et al., 2014). However, AMPA depends on the soils' physicochemical properties (Zhang et al., 2015), which was not the approach in the present study, as AMPA is a part of the glyphosate-herbicidal mechanism.

#### 4.3. Glyphosate phytotoxicity symptoms and its effects on fruit production

Chlorosis, defoliation, and leaf deformation were the main symptoms caused by glyphosate in citrus plants. Other symptoms such as internodes shortening, increased number of branches and leaves, and stunting were not observed even after App.#2. In a 5-yr field experiment, it was observed that citrus plants could stop (or diminish) the externalization of the glyphosate effects from a specific plant age; however, the plants continued to show severe decreases in growth and fruit yield, even in the absence of symptoms (hidden phytotoxicity) (Martinelli et al., 2022).

All glyphosate effects on citrus plants as a function of increasing doses impacted the fruit yield. These results agree with Martinelli et al. (2022). They recorded that doses of 2160 g ae ha<sup>-1</sup>, regardless of the number of applications, reduced fruit yield by 70%, 59%, 46%, 23%, and 34% (46% on average) during five consecutive agricultural years (from 2016/2017 to 2020/2021). In the present study, glyphosate was applied directly to the citrus leaves; as in the previously mentioned field experiment, the drift conditions were at least minimally controlled, suggesting that glyphosate drift was not the only way that this herbicide can cause disturbances in the shikimic acid pathway of citrus plants.

Although this study contrasts with the results of Gravina et al. (2009, 2012) and Matallo et al. (2010), the effects of glyphosate on citrus plants were explicit. The present study corroborates that the damage caused by the incorrect use of glyphosate in citrus can be assessed by analyzing *Shk* accumulation, photosynthesis, and the related parameters evaluated here. Even when the *Shk* accumulation was not directly correlated with some of them, it is known that glyphosate can impact the biosynthesis of several secondary compounds (Maeda and Dudareva, 2012), which must be better evaluated for citrus plants. Nevertheless, measuring the photosynthesis parameters was paramount to understanding the impact that *Shk* accumulation promoted. This study is the first report to demonstrate the glyphosate effects on the shikimic acid pathway, photosynthesis, and its correlations in citrus plants, including the effects on fruit production. It is of paramount importance that even nowadays, it is unclear how glyphosate leads to plant death. Some hypotheses still lead to protein depletion and additional C drainage, as the mechanisms leading to plant death may also be related to glyphosate's secondary or indirect effects on plant physiology (Gomes et al., 2014), demonstrated in the present study.

In addition, the results of this study are relevant because the various parameters were evaluated for two applications at a 240-d interval, i.e., the entire experiment lasted more than a year. However, most citrus growers make more than two applications per year at shorter intervals (Martinelli et al., 2017; Azevedo et al., 2020); therefore, the impact of glyphosate on citrus can be more severe under field conditions than what was verified in this study.

Moreover, another concern in the Brazilian herbicide industry is the unspecific information on many herbicide labels, whether due to a lack of initiative by manufacturers or regulatory agencies. As an example, the RoundUp Original® label in 2016 (MAPA – MINISTÉRIO DA

AGRICULTURA, PECUÁRIA E ABASTECIMENTO, 2016) recommended doses up to 1780 g ae ha<sup>-1</sup> for many weed species found in citrus orchards in the region of this study; however, it did not specify the culture or the maximum application frequency putting at risk the growers' orchards. This pattern is still followed by several glyphosate-based herbicides today (2022). A new glyphosate formulation (RoundUp Original Mais®) recommends a range of doses (up to 2160 g ae ha<sup>-1</sup>) for citrus orchards with a maximum of three applications per year; however, these recommendations are the same for other crops such as coffee, eucalyptus, and sugarcane (MAPA – MINISTÉRIO DA AGRICULTURA, PECUÁRIA E ABASTECIMENTO, 2021).

Therefore, integrated weed management (IWM) programs that include chemical and non-chemical methods should be implemented to reduce glyphosate dependency and adverse effects on citrus plants. The herbicides included must have different mechanisms of action. When glyphosate is used, the applied doses must be carefully addressed not to cause phytotoxicity; therefore, more detailed studies are required on the interaction between glyphosate, weeds, and perennial crops, such as citrus.

#### 5. Conclusions

The results of this study corroborate the hypothesis that glyphosate, mainly in doses higher than 360 g ea ha<sup>-1</sup>, damages citrus plants over time by its direct contact due to chronic disturbances in the shikimic acid pathway and the photosynthesis processes, even when the phytotoxicity is not visible (hidden phytotoxicity), on which there is a cumulative effect with consecutive glyphosate applications. These metabolic and physiological disturbances reduce the citrus fruit yield. However, further studies should be conducted to identify how the citrus plants regulate their photorespiration route to inhibit photooxidation when damaged by this herbicide and how different citrus varieties behave with their phytotoxicity.

#### Authors' contributions (CREDIT)

R.M.: Conceptualization; Supervision; Methodology; Formal analysis; Software; Data curation; Investigation; Writing – original draft; Writing – review & editing; L.R.R.Jr: Investigation; Supervision; Data curation; Writing – original draft; A.C.M.: Investigation; Supervision; Data curation; Writing – original draft; R.A.d.I.C and M.F.d.G.F.D.S: Investigation; Methodology; Formal analysis; Software; Data curation; Writing – original draft; Writing – review & editing; J.R.S.: Investigation; Methodology; Formal analysis; Software; Data curation; Writing – review & editing; R.M.B.: Methodology; Writing – original draft; Writing – Reviewing and Editing; P.A.M.: Supervision; Conceptualization; Methodology; Writing – original draft; Writing – Reviewing and Editing; D.M.Jr.: Funding acquisition; Supervision; Writing – Reviewing and Editing. F.A.d.A.: Project administration; Funding acquisition; Supervision; Conceptualization; Methodology; Resources; Data curation; Writing – original draft; Writing – Reviewing and Editing.

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#### Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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## Data availability

Data will be made available on request.

## References

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