Journal of Research High School

Vol. 2023 (2) 235 – 246
ISSN 2688-3651 [online]

An In Vitro Study on the Phytotoxic Effects of Glyphosate on *Spinacia oleracea*

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Received January 8, 2023; Revised July 17, 2023; Accepted, August 14, 2023

Abstract

Glyphosate is the most common non-selective herbicide to date. Evidence shows increasing glyphosate misuse and mishandling, prompting noticeable glyphosate residues on many non-targeted plants. This study investigates, in vitro, the phytotoxicity of 0.00 % (control), 0.05 %, 0.10 % and 0.20 % glyphosate v/v% concentrations on *Spinacia oleracea*'s photosynthetic rate (1/min) and total chlorophyll content (µg chlorophyll/g tissue) physico-chemical biomarkers. A large leaf tissue was subjected to each glyphosate concentration for 2.0 min, where 300 mg samples were taken pre- and post-glyphosate treatment for chlorophyll extraction and spectrophotometric quantification. Moreover, excess treated tissue were made down to 15 small identical disks for the indirect measurement of the photosynthetic rate via photosyn- thetic flotation technique. Our results indicate immediate adverse phytotoxic effects on both photosyn- thetic biomarkers. Short-term exposure to the lowest glyphosate concentration (0.05 %) had considerably decreased the photosynthetic rate from the control, reaching to the maximum of 71.4 % for the highest glyphosate concentration (0.20 %). Each glyphosate concentration resulted in a statistically significan ($p < 0.01$) lower total chlorophyll content than their initial values. All absolute total chlorophyll content percentage changes for all glyphosate concentrations were significantly different ($p < 0.05$) from the con- trol, yet 0.05 % and 0.10 % glyphosate concentrations' results show no significance in their difference at $\alpha = 0.05$. Observations revealed the possible 5-enolpyruvylshikimate-3-phosphate synthase competitive inhibition and the decrease in magnesium ion bioavailability for chlorophyll biosynthesis, calling for more caution when handling this chemical.

Keywords: Glyphosate, Spinacia oleracea, Competitive Inhibition, Photosynthesis, Chlorophyll, Chelating Agent, Shikimic Acid

1. Introduction

Glyphosate is a well-known non-selective herbicide that is effective at "managing incisive and noxious weeds" and used in various settings including "agricultural, residential and commercial settings" (Gomes et al., 2014; US EPA, 2014). However, in the hands of the uninformed, it may harm humans, plants, food and the environment in general if it is overused. Moreover, the main method of applying this chemical is by spraying (US EPA, 2014), which increases the chances of its transferal to non-targeted plants making them susceptible to its phytotoxic effects by the wind and/or water contamination (Gomes et al., 2014). With these different possible hazardous outcomes, this study examines the in vitro effects of increasing glyphosate v/v% concentration (0.00 %, 0.05 %, 0.10 % and 0.20 %) on the photosynthetic rate (# of floating disks/min) and Total Chlorophyll Content (*TCC*) (µg chlorophyll/g tissue) of *Spinacia oleracea* after a brief 2.0 min exposure time.

Photosynthesis is an important biochemical process that most plants undergo. In it, plants convert inorganic substances (water and carbon dioxide) to organic ones (glucose) through a series of steps; some require the presence of light (light-dependent reactions) while others do not (light-independent reactions) ("Overview of Photosynthesis – Biology 2e", n.d.). It might seem like both reactions occur individually; however, they are highly dependent on one another. For example, the Calvin cycle - a cycle of light-independent reactions that occurs in the stroma of the chloroplast - requires Adenosine Triphosphate (ATP) and Reduced Nicotinamide Adenine Dinucleotide Phosphate

Vol. 2023 (2) 235 – 246
ISSN 2688-3651 [online]

(NADPH) to reduce 3-phosphoglyceric acid (3-PGA) into Glyceraldehyde-3-Phosphate (G3P) and to regenerate Ribulose-1,5-Bisphosphate (RuBP) in order to have a continuous cycle yielding glucose (Molnar & Gair, 2015) - a great energy source.

In order to attain these molecules (ATP and NADPH), light-dependent reactions must occur (summarized in Figure 1) (Arnon, 1971). This takes place in the thylakoid membrane where:

(a) A Photosynthetically Active Radiation (PAR) (between 400 nm and 700 nm) strikes a pigment molecule (e.g., chlorophylls) in Photosystem II (PSII) exciting its electron and initiating a series of electron movements until it donates to the primary electron acceptor, Pheophytin (Pheo), in its P680 reaction center (Allakhverdiev et al., 2010). To replace this missing electron, an electron is taken from a water molecule as it breaks down to two hydrogen cations, two electrons and one oxygen atom by photolysis as seen below (Arnon, 1971). This whole process is called photoactivation.

$$H_2O \xrightarrow{h\nu} 2H^+ + 2e^- + \frac{1}{2}O_2$$

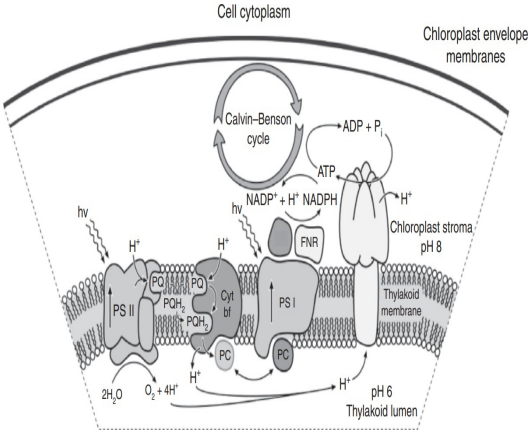

Figure 1. A diagram showing the cross section of a chloroplast and a thylakoid membrane adopted from (Masojídek et al., 2013). It also shows the steps that occur in the light-dependent reaction including photoactivation, photophosphorylation and the reduction of $NADP^+$.

(b) Pheo is then oxidized as its electron flows through multiple membrane proteins making up Electron-Transport-Chain (ETC) that actively pumps H^+ into thylakoid space with the energy released by each successive movement of electron through them. This creates a higher $[H^+]$ in the thylakoid space than in the stroma, causing diffusion of them, down the electrochemical gradient, through ATP synthase, and generating energy by chemiosmosis to phosphorylate Adenosine Diphosphate (ADP) to ATP. This whole process is called photophosphorylation.

(c) The electron from PSII will act as a source for the missing electron in Photosystem I (PSI); as the electron of its pigment molecules will also get excited and ultimately donated to its primary electron acceptor, chlorophyll A0 ("Light-dependent reactions (photosynthesis reaction) (article)", n.d.). A0 will then get oxidized and its electron will either flow back through the first ETC (if light is not the limiting factor) in a process called cyclic photophosphorylation (Arnon, 1971), or donated to the second ETC that results in reduction of Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$) to NADPH by the addition of two electrons and a H^+ by the last enzyme, Ferredoxin-$NADP^+$ reductase (FNR) (Arnon, 1971; Mulo, 2011).

As stated, glyphosate (N-(phosphonomethyl)glycine) is a non-selective herbicide that seems to indirectly effect the light-dependent reaction outlined earlier (Gomes et al., 2014). It has been long believed that the main reason behind plant death after being exposed to glyphosate is due to its inhibitory effect on 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Gomes et al., 2014). EPSP synthase is an important enzyme in the shikimate pathway that is responsible for catalyzing the conversion of Shikimate-3-Phosphate (S3P) to the sixth intermediate, EPSP, by addition of Phosphoenolpyruvate (PEP) to its molecule and the release of Inorganic phosphate (P_i) as seen in Figure 2 (de Souza & Sant'Anna, 2008). However, some scholars believe that their death is for the secondary indirect negative effects it has on several important components of the light-dependent reaction (Gomes et al., 2014). For example, the result of inhibiting EPSP synthase is the decrease in the biosynthesis of quinones (Gomes et al., 2014) - a set of e^-/H^+ carriers (El-Najjar et al., 2011; Nowicka & Kruk, 2010). This is because isoprenoid quinones are biosynthesized through metabolic pathways that originally start with aromatic amino acids that are the end products of the shikimate pathway (Nowicka & Kruk, 2010; Tzin et al., 2012). Thus, the biosynthesis of Plastoquinone (PQ) - an important electron carrier between PSII and Cytochrome-b_6f-complex (Cytb_6f) in the light-dependent reaction derived from

Vol. 2023 (2) 235 – 246
ISSN 2688-3651 [online]

tyrosine (Nowicka & Kruk, 2010) - will be inhibited, causing electrons to accumulate, and thus, decreases the need of electrons from the photolysis of water, and in general, decreases the photosynthetic rate of the plant.

Not only does this competitive inhibitor (competes with PEP for EPSP synthase's active site) limit the pro- duction of PQs, it also negatively affect chlorophyll biosynthesis (Gomes et al., 2014). Chlorophyll, as mentioned earlier, plays an important role in the first stages of the light dependent reaction. It consists of a porphyrin ring with magnesium as the central metal atom of its structure (Sapkota, 2020). This central metal atom is largely responsible for giving chlorophyll its color and role in photosynthesis (Farhat et al., 2016).

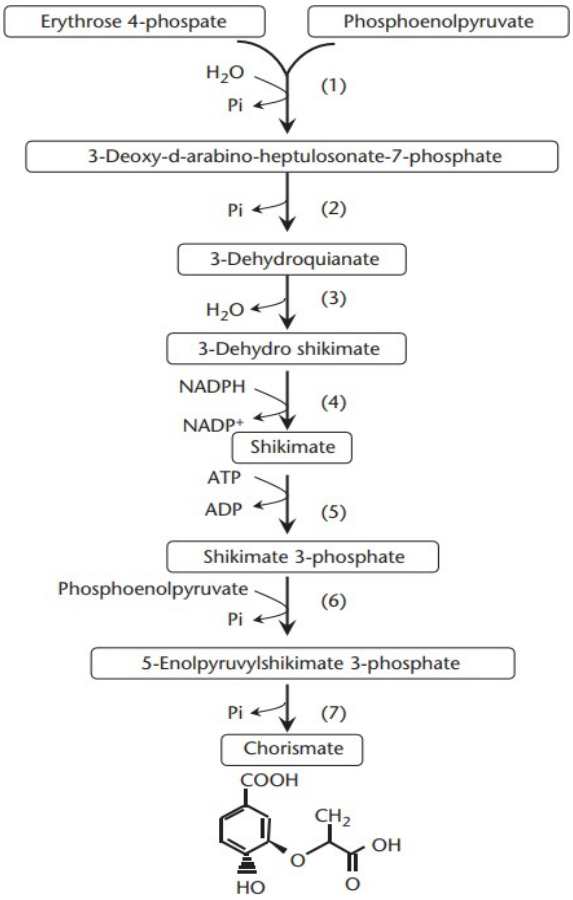

Figure 2. A flowchart adopted from (Tzin et al., 2012) that shows all seven intermediates of the shikimate metabolic pathway. EPSP synthase is responsible for the sixth (6) intermediate.

Magnesium ion is added to Protoporphyrin IX (PPIX) by the magnesium-chelatase in the early stages of chlorophyll biosynthesis (Figure 3) (Farhat et al., 2016), however, without magnesium, this obviously will not occur. Glyphosate is known as a "potent chelator for minerals" and magnesium is one of those minerals that it binds to ("Glyphosate, a chelating agent—relevant for ecological risk assessment?", n.d.). Therefore, exposing plants to glyphosate may result in magnesium deficiency, inhibiting the biosynthesis of chlorophyll and leading to lower photosynthetic rates.

As a result, it is hypothesized that as Glyphosate Concentration ([GP]) increases, *Spinacia oleracea*'s photosynthetic rate (# of floating disks/min) and *TCC* (µg chlorophyll/g tissue) will decrease due to (a) the in-crease probability of glyphosate binding to EPSP synthase instead of PEP, which ultimately leads to the decrease in the availability of aromatic amino acids needed for the biosynthesis of PQs that are responsible for the movement of electrons in the light-dependent reaction; (b) the increase probability of forming magnesium-glyphosate chelates that decreases the availability of magnesium needed for the biosynthesis of chlorophylls essential for photoactivation. To investigate these effects, the methodology of this experiment was based on photosynthetic flotation technique and spectrophotometry to measure the photosynthetic rate and TCC of the plant, Spinacia oleracea, respectively.

Photosynthetic flotation (or floating leaf disk) experiment is an indirect way of measuring the photosynthetic rate of mainly terrestrial plants such as *Spinacia oleracea*. It exploits the decrease in the buoyancy of the small leaf disks as oxygen generation from photosynthesis' light-dependent reaction (the photolysis of water) accumulates in the spaces found in its spongy mesophyll layer. These spaces were initially filled with a carbon-containing solution of a specific concentration by "force". Doing it by "force" involves creating a pressure difference "sucking" oxygen out of the leaf disks and then releasing the pressure to "push" the sodium bicarbonate ($NaHCO_3$) solution through its stomata and into the spongy mesophyll layer causing the decrease in the disk's buoyancy to the point of sinking in water. Thus, the higher the number of floating disks are at a given time, the faster the rate of photosynthesis it indirectly indicates as the oxygen replaces $NaHCO_3$ in these spaces.

Vol. 2023 (2) 235 – 246
ISSN 2688-3651 [online]

Spectrophotometry is a technique used to measure the light absorbance and/or transmittance through a solute containing liquid of unknown concentration ("Spectrophotometry", n.d.). This is done using a spectrophotometer that passes a beam of light of chosen wavelength through a liquid sample (held inside a cuvette) and onto a photocell (a light detector) ("Spectrophotometry", n.d.). Therefore, as solute concentration increases, more of the specific wavelength beam of light is absorbed giving us lower light transmittance value but higher absorbance value.

In this case, a spectrophotometer will be used on a chlorophyll-containing solution after extraction using 90 % acetone following a slightly modified (Su et al., 2010) procedure. Absorbance readings at 645 nm and 663 nm is needed to calculate the *TCC* (µg chlorophyll/g tissue) using ("3. METHODOLOGY", n.d.)'s equation (Equation 1).

$$TCC = \frac{V(20.2(A_{645})+8.02(A_{663}))}{M} \quad (1)$$

Where,
TCC : the Total Chlorophyll Content in µg chlorophyll/g tissue
V : final volume of the extract in mL
Ax : the absorbance value at Xnm
M : the mass of the leaf tissue in g

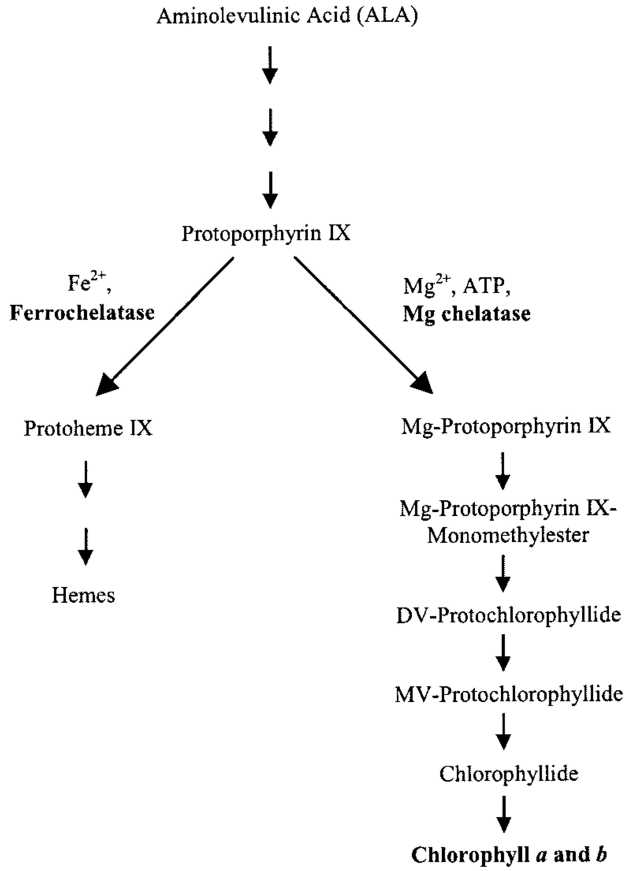

Figure 3. A flowchart adopted from (Rissler et al., 2002) that shows the metabolic pathway in which chlorophylls are synthesized.

2. Materials and Methods

2.1 Variables

Independent Variables

Glyphosate v/v% concentrations (0.00 %, 0.05 %, 0.10 % and 0.20 %) and the specific time intervals in which measurements were taken. For the photosynthetic flotation, counting the number of floating leaf disks were done after every 30.00 s for 56.0 min. For the *TCC*, sampling were taken before and after exposure to the [GP] in question for 2.0 min.

Dependent Variables

The photosynthetic rate (# of floating disks/min) of *Spinacia oleracea* measured by the rate in which the leaf disks float using the photosynthetic flotation technique and the *TCC* (µg chlorophyll/g tissue) measured spectrophotometrically.

Controlled Variables

The controlled variables of this experiment are listed in Table 1.

Vol. 2023 (2) 235 – 246
ISSN 2688-3651 [online]

Table 1. The controlled variables in different parts of the experiment (Photosynthetic Flotation (PF), Chlorophyll Quantification (CQ) and both), how they are controlled and why

Parts	Controlled Variables	How and Why Variable is Controlled
Both	Type of plants	Different types of plants have different concentrations of photosynthetic pigments, and thus, different photosynthetic rates. Therefore, Spinacia oleracea was only used throughout the experiment.
	Glyphosate exposure time	Giving a trial longer glyphosate exposure time than the others will damage its leaf more and ultimately give false results. Thus, all trials will have the leaves exposed to the glyphosate v/v% concentration in question for 2.0 min.
PF	Size and number of leaf disks	The larger the disk is, the more photosynthesising cells it has, the faster the photosynthetic rate. However, it also has a greater mass than the smaller disks so it may take longer to float. Furthermore, more disks will increase the dependence on the dissolved carbon in the solution. Thus, all disks are 5.0mm in diameter and limited to 15 in number.
	Volume and concentration of $NaHCO_3$	Dissolved $NaHCO_3$ will act as a source of carbon needed for photosynthesis. Since carbon dioxide is a limiting factor of photosynthesis, increasing or decreasing the volume and/or concentration of $NaHCO_3$ will effect the results until a certain point. Thus, excess 40.0mL of 0.1% (w/v%) $NaHCO_3$ is used for all trials to prevent it being a limiting reagent.
	Net pressure on leaf disks	The deeper the disks are, the higher the pressure (from the above liquid and the atmospheric pressure) acting upon the disks. This will decrease the size of the air bubbles making it harder for the disks to float. Thus, the volume of the liquid (0.1% (w/v%) $NaHCO_3$ in this case) is always 40.0mL and the beaker is the same for all trials.
	Light intensity	Light intensity is one of the limiting factors of photosynthesis which will increase or decrease its rate. Thus, the distance away from the light source (3.00 cm) and its power (23 W) are constant throughout the experiment.
	Total time of the experiment	Extending the total time of the experiment for one trial than the others will allow more disks to float giving false results. Thus, the total time of all trials is 56.0 min.
CQ	Mass of the leaf samples	Taking different masses of leaf samples for chlorophyll quantification will give false results if not accounted for. Thus, all leaf samples (both initial and final) in all trials are 300 mg in mass.
	Homogenization time and acetone concentration	Giving a trial less homogenization time may indicate lower total chlorophyll concentration. Moreover, the higher the concentration of acetone, the more chlorophyll is dissolved leading to higher results (Su et al., 2010). Thus, homogenization time and acetone concentration is fixed to 1.0 min and 90 %, respectively, for all trials.
	Final extract volume	Different final extract volume will give different absorbance values, and thus, false/unrepresentative results. Thus, the final extract volume was fixed to 10.0mL for all trials.
	Differential centrifugation speed and time	Centrifugation is needed to filter out organelles from the extract. This requires a specific speed and time dependent on that speed. Thus, the centrifugation speed and time were fixed to 3000 rpm and 10.0 min, respectively, for all trials.
	Absorbance wavelength	Equation 1 requires absorbance value of the extract at 645 nm and 663 nm. Changing the wavelength will give different values depending on chlorophyll's absorption spectrum. Thus, the wavelengths are fixed on the required ones for all trials.

Monitored Variables

The temperature in which the photosynthetic flotation experiment was taken place was monitored to be between 25.00 °C and 31.00 °C inclusive.

Vol. 2023 (2) 235 – 246
ISSN 2688-3651 [online]

2.2 Experimental Materials

Healthy *Spinacia oleracea* leaves where purchased from a local retailer the same day as the experiment was performed. A 23 W OSRAM DULUX Mini Twist was used as a light source and Eppendorf™Centrifuge Model 5702 was used for centrifugation. A 752n Lab Optical Instrument Single Beam UV Vis Spectrophotometer was the spectrophotometer of choice.

2.3 Photosynthetic Flotation and Sampling

300 mg of a large *Spinacia oleracea* leaf was taken as an initial chlorophyll sample and placed inside a small labeled dark container. The rest of the leaf was submerged under a 100.0 mL solution of the glyphosate v/v% concentration in question for 2.0 min. It was then dried and superficially washed with distilled water to remove any leftover solution on it. Another 300 mg of the leaf was removed as a final chlorophyll sample and placed in its designated container. Both 300 mg samples are needed for chlorophyll extraction and quantification (subsection 2.4).

What was left from the leaf was cut, with veins avoided, into 15 small circular disks of diameter 5.0 mm. Disks were then placed inside a 25.0 mL needle-less syringe containing 10.0 mL 0.1 % (w/v%) $NaHCO_3$ solution. The syringe was vigorously shaken and a combination of pulling and pushing the plunger were used while the opening tip was closed. Occasionally, the tip was opened to release the trapped air that had been forced out of the leaf tissue. This process ended once the were-floating 15 disks all sank. The whole solution with the 15 sunken disks were placed inside a small beaker and made up to 40.0 mL using the same $NaHCO_3$ solution used earlier.

The beaker was positioned 3.00 cm away from a 23 W light source and measurements started immediately. Counting the number of floating leaves was done after every 30.00 s. The temperature was monitored every 2.0 min. The solution was mixed gently after every 10.0 min to move any stuck/adhered disks. All measurements where conducted for 56.0 min. This was repeated four other times to have a total of five trials at each glyphosate v/v% concentration.

2.4 Chlorophyll Extraction and Quantification

The initial 300 mg leaf sample was further cut down into smaller pieces. It was then homogenized for 1.0 min with10.0 mL of 90 % acetone using a mortar and pestle. The homogenate was filtered and poured into a labeled graduated centrifuge test tube. The homogenate was then made up to 10.0 mL using the same acetone used earlier. This was repeated on the other leaf sample to give us a total of two labeled centrifuge tubes. These were then centrifuged at 3000 rpm for 10.0 min and the supernatant of each were taken for absorbance measurement at 645 nm and 663 nm. All steps were superficially summarized in Figure 4 for *one* trial of *one* glyphosate increment.

2.5 Risk Assessment

Safety Issues

No plastic equipment must be used when dealing with an acetone containing liquid; as acetone (especially when concentrated) can "damage the plastic's surface, softening it, smearing it, or even dissolving the plastic [itself]" ("How Acetone Affects Certain Plastics", 2020). Moreover, acetone is a "flammable liquid [and vapour], [serious] eye irritant" and "may cause drowsiness or dizziness" upon inhalation ("SAFETY DATA SHEET Acetone", 2015). Thus, the use of acetone must be in a fully ventilated room away from sparks or any heat emitting instruments. Furthermore, protective clothing (e.g., masks, lab coats, etc.) must be worn.

Similarly, proper protective eye-wear, clothing and gloves must be used when dealing with glyphosate ("MATERIAL SAFETY DATA SHEET Glyphosate 5.4", 2007). Glyphosate may cause "slight eye [and skin] irritations" which may worsen the more concentrated the product is. It is "harmful if inhaled" and slightly toxic "if small amount is swallowed" ("MATERIAL SAFETY DATA SHEET Glyphosate 5.4", 2007). Thus, a properly ventilated room is also advised ("MATERIAL SAFETY DATA SHEET Glyphosate 5.4", 2007).

Vol. 2023 (2) 235 – 246
ISSN 2688-3651 [online]

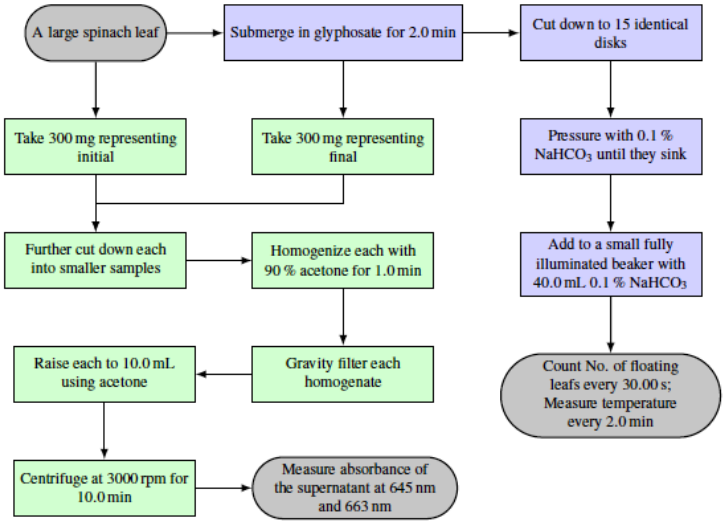

Figure 4. A flowchart outlining the key steps in the methodology used for one trial of one glyphosate increment. This includes both photosynthetic flotation and sampling (blue) as well as chlorophyll extraction and quantification (green).

Operating a centrifuge must be under the supervision or done by an expert. The centrifuge must be filled with an even number of centrifuge tubes all of similar mass and directly opposite each other. An unbalanced centrifuge at high speeds may lead to damages, injuries or even deaths. Thus, eye protection must be used when near a running centrifuge.

Environmental Issues

While acetone is "non toxic to aquatic organisms", "readily biodegradable in aerobic systems" and "poorly absorbed onto soils or sediments", it should "[not] enter drains and watercourses" ("SAFETY DATA SHEET Acetone", 2015). This also applies to glyphosate as it "[must] not contaminate water" as it "can result in oxygen depletion due to decomposition of dead plants" causing nearby fish to suffocate ("MATERIAL SAFETY DATA SHEET Glyphosate 5.4", 2007). Moreover, no excessive material consumption was used. The experiment was designed to minimize waste as much as possible while still allowing it to proceed.

2.6 Statistical Analysis

Where suitable, t-Test was conducted on mean experimental values at 95 % and 99 % confidence levels. Moreover, one-way ANOVA test was use with Tukey HSD post-hoc test at 95 % confidence level. H_0 = mean values of the variable(s) in question are *not* statistically significant at $\alpha = 0.05$ and/or $\alpha = 0.01$ where differences may have been the result of random chance; H_a = mean values of the variable(s) in question are statistically significant at $\alpha = 0.05$ and/or $\alpha = 0.01$ where differences may *not* be the cause of random chance.

Table 2. Paired two-tailed t-Test on the Initial and Final *TCC* of each glyphosate concentration (%)

Glyphosate Concentration (%)	*t*	*p*	$p < 0.05$?	$p < 0.01$?
0.00	2.90	0.0441	yes	no
0.05	17.3	6.53×10^{-5}	yes	yes
0.10	5.73	0.00460	yes	yes
0.20	16.7	7.55×10^{-5}	yes	yes

The statistical tests used are found in Table 2 and Table 3 below. Note that with $k = 4$ and $df_{within} = 16$, $Q_{0.05} = 4.046$ is the critical value at $\alpha = 0.05$ for Tukey HSD test. If $Q > Q_{0.05}$, then it is statistically significant at $\alpha = 0.05$. Similarly, $p < 0.05$ and $p < 0.01$ show significance at $\alpha = 0.05$ and $\alpha = 0.01$ respectively.

Table 3. Tukey HSD post-hoc test on the absolute % changes in *TCC*

A(%)	&	*B*(%)	*diff*	*n*(*A*)	*n*(*B*)	Standard Error	*Q*	$Q > Q_{0.05}$?
0.00	&	0.05	17.2	5	5	2.45	7.00	yes
0.00	&	0.10	26.3	5	5	2.45	10.7	yes
0.00	&	0.20	38.2	5	5	2.45	15.6	yes
0.05	&	0.10	9.12	5	5	2.45	3.71	no
0.05	&	0.20	21.0	5	5	2.45	8.55	yes
0.10	&	0.20	11.9	5	5	2.45	4.84	yes

Note—Done after insuring that H_0 is rejected in one-way ANOVA ($p = 6.9302 \times 10^{-8}$) at $\alpha = 0.05$; $Q_{0.05} = 4.046$; *A* and *B* are glyphosate concentrations.

Vol. 2023 (2) 235 – 246
ISSN 2688-3651 [online]

3. Results and Discussion

The results of this experiment suggests that glyphosate has a prominent effect on both the light-dependent reactions (oxygen gas generation) and *TCC* of the terrestrial plant *Spinacia oleracea*. These effects get more pronounced as the concentration of the substance increases.

Take for instance the mean number of floating leaf disks from the photosynthetic flotation experiment (Figure 5). In line with the hypothesis, the mean number of floating leaf disks in most time intervals are higher in that of the control than the ones treated with glyphosate. Increasing at an exponential rate, and on average, 92 % of the total 15 leaf disks were floating by the end of the experiment (56.0 min) in the control. On the other hand, exposure to as low as 0.05 % [GP] gave a value ((8.2 ±1.3)) surprisingly 41 % lower than the control ((13.8 ±1.1)) at that time interval. This decrease increased to 59 % for 0.10 % [GP] ((5.6 ±1.3)) and 72 % for 0.20 % [GP] ((3.8 ± 2.5)).

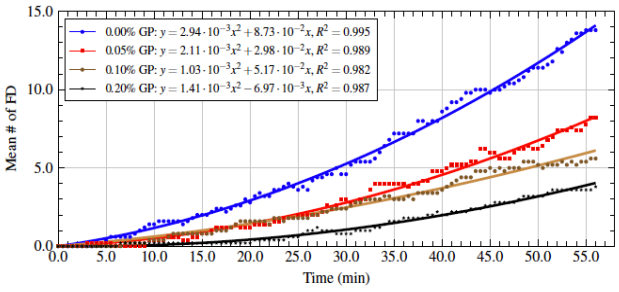

Figure 5. The average (n = 5) number of floating leaf disks (FD) after each 30.00 s time intervals. Polynomial functions were fitted to each glyphosate (GP) concentration. The standard deviations were omitted for better visualisation.

Moreover, the mean rate of change in number of floating leaf disks (a.k.a. the photosynthetic rate) between zero and 56.0 min show a decreasing trend with increasing [GP] (Table 4). This seems to be in an exponential decay fashion (R^2 = 0.987) with a clear indication of a plateau starting from, and unexpectedly speaking, 0.20 % [GP] (Figure 6). Yet, as expected, this concentration shows the largest percentage change (71 %) from the photosynthetic rate of the control (0.252/min) than the others have. However, the difference between adjacent rates is the highest (0.104/min) between 0.05 % [GP] and the control, indicating an immediate and strong adverse effects.

The general trend of the above results are as expected and demonstrated in several published studies. With exposure to glyphosate, the production of aromatic amino acids decreases as it inhibits EPSP synthase from catalysing the sixth intermediate of the shikimate pathway (de Souza & Sant'Anna, 2008; El-Najjar et al., 2011; Gomes et al., 2014; Nowicka & Kruk, 2010; Tzin et al., 2012). As a linear metabolic pathway, interrupting any of its intermediates would stop it completely (Tzin et al., 2012). Doing so decreases the biosynthesis of PQs needed for electron movement in the light-dependent reaction (El-Najjar et al., 2011; Nowicka & Kruk, 2010; Tzin et al., 2012). Causing electrons to accumulate, the photolysis of water decreases as indicated by the decrease in the rate of oxygen generation. Our results show a significant decrease in this area indirectly measured by the rate of floating disks after application with glyphosate. As this is a competitive inhibition, increasing [GP] increases the frequency of successful collisions with EPSP synthase's active site (Gomes et al., 2014; Nowicka & Kruk, 2010). This explains why our results show decreasing rates with increasing inhibitor concentration, consistent with present studies.

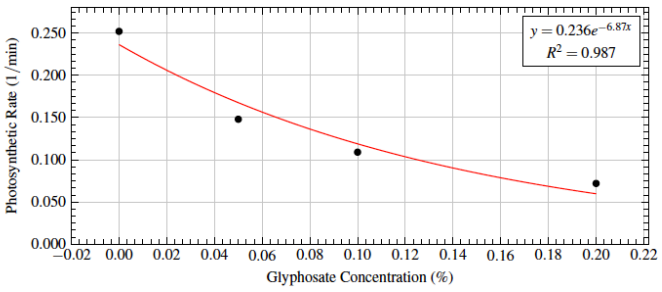

Figure 6. The relationship between the photosynthetic rate (indirectly measured via the mean rate of change in number of floating leaf disk) and glyphosate concentration. A best fit line (-) is presented. Data from Table 4.

However, plateauing at such a low concentration is somewhat unexpected given that glyphosate is commercially sold at around 50 % concentration. Yet publications show similar effects on different plants by using different techniques of measurement such as the direct measurement of the activity of PSII, the electron transport rate through it and/or shikimic acid content (Huang et al., 2012; Meloni et al., 2022). While our study show a decrease in photosynthetic rate post-exposure to glyphosate, some studies did not (Cedergreen & Olesen, 2010; Khan et al., 2020). One of which is (Khan et al., 2020) whose results

Vol. 2023 (2) 235 – 246
ISSN 2688-3651 [online]

seems to indicate the exact opposite effects. In all of the [GP]s, post- photosynthetic rate was higher than the ones pre-exposed to glyphosate up until higher concentrations; where effects are similar to ours. Both (Khan et al., 2020)

and (Cedergreen & Olesen, 2010) attempted to explain such findings by the "increased efficiency of CO_2 fixation" upon exposure to low-doses of [GP]s, but such mechanism requires further research as they suggested.

Yet nonetheless, our results indicate a clear, strong and prominent effect of glyphosate on the photosynthetic rate of *Spinacia oleracea* at low-doses and limited exposure time. Differences in time of sampling as well as the type, size and age of the plant may have led to such differences compared to (Khan et al., 2020) and (Cedergreen & Olesen, 2010). Moreover, their "low-doses" in their in vivo experiments may be considered "high-doses" in our in vitro investigation with leaf tissue used as small as 5.0 mm in diameter. Lastly, our indirect measurement of the photosynthetic rate could be a factor aiding in this disparity. This is also a limitation of our experiment preventing us from determining the exact causes of our quantitative observations. These limitations also extend to the lack of statistical tests and high standard deviation values, which may be caused by slight temperature differences between insufficient amount of trials, adherence of leaf disks to the bottom of the beaker, leaf disks held by the surface tension of water and respiration that consumes oxygen and decreases the buoyancy of the disks. All limitations may have contributed to the fluctuation in number of disks at any given time interval and between trials. If not bounded to the lack of apparatus, oxygen and carbon dioxide sensors could be used and more trials could be conducted to allow for statistical tests to be done and decreases the mentioned limitations specifically for the photosynthetic flotation experiment.

Table 4. The mean rate of change in the number of floating leaf disks between zero and 56 min for each glyphosate concentration (%)

Glyphosate Concentration (%)	Mean Rate of Change in [0, 56] (1⁄min)*
0.00	0.252
0.05	0.148
0.10	0.109
0.20	0.072

Note—There are no standard deviation values and statistical tests due to the way rates were obtained. * The photo- synthetic rate.

Table 5. The mean Initial and Final Total Chlorophyll Content (*TCC*, in µg⁄g) and the mean absolute % change of each glyphosate concentration

Glyphosate Concentration (%)	Mean *TC* Initial	*C* (µg⁄g) Final	Mean Absolute % Change
0.00	448.7 ± 7.2	445.1 ± 5.6	0.8 ± 0.6a
0.05	441.2 ± 14.5	362.0 ± 21.5	18.0 ± 2.6b
0.10	428.8 ± 27.9	311.2 ± 30.4	27.1 ± 9.2b
0.20	438.3 ± 9.5	267.6 ± 25.4	39.0 ± 5.3c

Note—Numbers represent Mean ± Standard Deviation for a sample set of data (n = 5). Letters indicate significant difference at $p < 0.05$ by one-way ANOVA with Tukey HSD post-hoc test. Statistical tests results in Table 3.

In regards to the absolute percentage change in the *TCC* of *Spinacia oleracea* (Figure 7), clear differences can be concluded between [GP]s. However, and contrary to our expectations, qualitative findings seem to indicate little to no color changes. Yet, quantitatively speaking, and similar to the photosynthetic rates, exposure to the lowest [GP] gave an astonishing 18.0 % change in *TCC* that is significant at α = 0.05 (Table 5). Higher concentrations gave higher percentage changes all of which are statistically significant at α = 0.05. However, and with a high standard deviation value (9.2 %), the absolute percentage change at 0.10 % [GP] is not statistically significant from its lower adjacent concentration at the same confidence level. The use of plastic cuvettes could have led to such a problem; as acetone was able to deform the cuvette's structure leading to the fracturing of the beam of light and falsely analyzed as absorbance. A cuvette of other material should be used.

As a chelator of minerals, glyphosate decreases the bioavailability of magnesium ions needed for chlorophyll synthesis by forming magnesium-glyphosate chelates that prevents magnesium-chelatase from catalyzing the addition of magnesium to PPIX (Farhat et al., 2016; Gomes et al., 2014; Guo et al., 2015; Huang et al., 2012). With higher [GP]s, the probability of forming the chelates and their quantity increases as indirectly indicated by our results (Figure 7) and (Huang et al., 2012). (Huang et al., 2012)'s results took days, which show that the damage done by glyphosate is rather progressive than immediate. Nonetheless, we can 95 % confidently conclude that the addition of glyphosate is the factor behind the decrease in *TCC*, which is more significant at higher [GP]s. This may contribute to the decrease of

Vol. 2023 (2) 235 – 246
ISSN 2688-3651 [online]

photosynthetic rates mentioned previously; as such pigments are needed in the initial stages of the light-dependent reaction (photoactivation), without which, the photolysis of water decreases and so does oxygen generation.

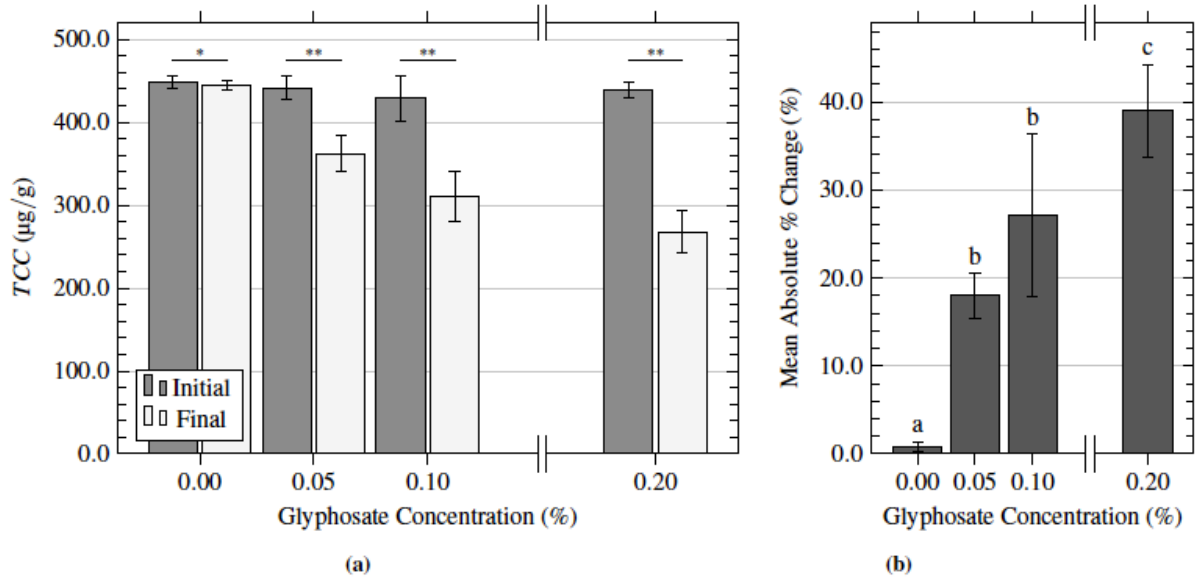

Figure 7. (a) The initial and final Total Chlorophyll Content (TCC) after exposure to each glyphosate concentration. Paired two-tailed t-Test was used to indicate significant difference at $p < 0.05$ (*) and $p < 0.01$ (**) (Table 2). (b) The average absolute % change in TCC after exposure to each glyphosate concentration. Letters indicate significant difference at $p < 0.05$ by one-way ANOVA with Tukey HSD Test as a post-hoc test (Table 3). Error bars in both (a) and (b) are of the standard deviation values with $n = 5$. Data from Table 5.

4. Conclusion

This study was able to determine the in vitro effects of increasing [GP] on both the photosynthetic rate and *TCC* of *Spinacia oleracea*. With prominent and strong effects even upon exposure to the lowest [GP] (0.05 %), it was clear and statistically significant that glyphosate is responsible for the changes caused to both dependent variables. Increasing [GP] also attributed to the decreases found in both dependent variables, which supports our hypothesis and present detailed studies. Such causes may be explained by the inhibitory effect of this chemical on EPSP synthase, the nature of this inhibition and the decrease in magnesium ion bioavailability needed for chlorophyll synthesis. With some dissimilarities in photosynthetic rates (inverse effects) and qualitative evidence (no notable color changes) with other publications, it may be merely the cause of our experimental limitations, differences in plant, exposure time, sampling time and the overall way our experiment was designed. Yet, with nature in mind, this study was able to generate conclusions in a way that minimizes harm to the environment and the ecosystem, and consistent, in most cases, with cited articles. The drawn conclusions could be of great interest to the users of this herbicide, increasing caution when spraying near similar non-targeted plants. While our study investigated the immediate effects of this chemical on two aspects of this organism, hormonal changes was not in mind and further research should be held investigating the reversibility of mentioned effects and the factors that decreases the mortality rate upon accidental exposure to this chemical.

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