The Effect of The Herbicide of Glyphosate (Nphosphonomethyl Glycine) On Photosynthetic Pigments And Photosynthetic Activity of Basil (*Ocimum Basilicum* L.) By Laser-Induced Chlorophyll Fluorescence Spectroscopy

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Research Article

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Abstract

The aim of this study was to investigate the effect of short and long term exposure to N-(phosphonomethyl)glycine (glyphosate) on the photosynthetic activity of *Ocimum basilicum* L. (basil) plants. Photosynthetic pigment content, chlorophyll fluorescence, and laser-induced chlorophyll fluorescence spectra of basil plants treated for 30, 50, or 70 days with either 0, 1, 2, or 3 ml/L (H₂O) glyphosate were measured. The ratio of the two fluorescence intensity maxima was calculated by evaluating curve-fitted parameters. Findings revealed that after 30 days of treatment, 1 ml/l (H₂O) of glyphosate stimulated, whereas 2 and 3 ml/l (H₂O) of glyphosate inhibited, photosynthetic activity of basil plants. In contrast, all tested concentrations of glyphosate became inhibitory after 50 and 70 days of treatment. This study clearly showed that at high concentrations (> 1 ml/l (H₂O)), glyphosate is highly toxic to basil. This finding may be helpful for determining the optimal dose of glyphosate in agricultural practices.

1. Introduction

As is known, the chlorophyll fluorescence measurements provide a technique for monitoring photosynthetic processes. And as that this technique depends on the observation that chlorophyll fluorescence depends in large part on the capacity of photosystem II to stably separate charges between P₆₈₀, the primary donor, and Q_A, the primary quinone acceptor, of the reaction center. When Q_A is oxidized, the reaction center is able to utilize the light energy harvested by the antenna system for charge separation and the fraction of excitation lost to fluorescence is low, giving rise to low fluorescence yields. In contrast, when Q_A is reduced, the reaction center is unable to stably separate charges and the fraction of excitation lost to fluorescence is high, giving rise to the maximum fluorescence yield. The recent introduction of highly sensitive charge-coupled device cameras has enabled the development of instrumentation that can image chlorophyll fluorescence in cells, leaves, and plants [1, 2].

While glyphosate and its formulations such as Roundup have been approved by regulatory bodies, worldwide concerns about their effects on humans and the environment persist[3]. Studies on the phytotoxic effects of organophosphorus insecticides on phytoplankton have indicated that this type of toxicant reduces growth rates and inhibits chlorophyll (Chl), protein, and carbohydrate biosynthesis [4, 5]. Dimethoate has been shown to induce enhancement of respiratory O₂ consumption in cyanobacteria [5, 6] and reduce PS II activity and photophosphorylation in *Synechocystis* cells, inhibiting photosynthetic electron transport [6, 7]. Additional reports suggest that dimethoate negatively affects electron transport between PS II and PS I at the level of plastoquinone (PQ) pool [5, 8], which can increase PS II fluorescence and reduced CO₂ fixation [7]. At elevated concentrations, dimethoate modifies the photosystem fluorescence of green algae and cyanobacteria [4, 8, 9].

Chlorophyll fluorescence exhibits two maxima at 685 nm and 730 nm [10]. The shape of the Chl fluorescence spectra and the value of the fluorescence intensity ratio (FIR) at these two fluorescence maxima (F_{685}/F_{730}) depend upon the Chl content of a given leaf [11]. At low Chl levels, the fluorescence emission spectrum only shows one maxima at 685 nm, with a shoulder near 730 nm. Alongside chloroplast and thylakoid multiplication, which proceeds during leaf development and greening, there is a massive accumulation of light-harvesting Chl a/b pigment-containing proteins, which mainly function in light absorption. This both increases the light absorption of leaves and the re-absorption of the emitted fluorescence. Thus by increasing Chl content, the shortest wavelength fluorescence becomes increasingly suppressed due to re-absorption of the emitted fluorescence by Chl, developing the shoulder at 730 nm into a second maximum [12] and decreasing the F_{685}/F_{730} ratio [10]. Since the Chl fluorescence ratio F_{685}/F_{730} is dependent on Chl concentration, this ratio can be used to monitor changes in Chl content during leaf development [13], autumnal Chl breakdown [14], re-greening of yellowish leaves [5], natural and anthropogenic stress, and damage [15, 16].

2. Experimental

2.1 Samples:

Plants were purchased from Riyadh nurseries in 2016-2017. They were placed in a park in the Physics and Astronomy Department at King Saud University and were regularly cleaned. The effects of glyphosate were analyzed using plant leaves after 30, 45, and 70 days of treatment. The plants were sprayed with different concentrations of glyphosate beginning after one week of glyphosate purchase and with 5 replicates per condition and 60 replicates per experiment. Levels of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids were determined spectrophotometrically [17]. After treatment, the topmost fully expanded leaves were weighed and dipped in 85% (v/v) aqueous acetone to extract chlorophyll pigments. The resulting supernatant was centrifuged at 4000 rpm for 10 min and diluted with 85% aqueous acetone to achieve a suitable concentration for spectrophotometric measurements. Absorbances at 452.5 nm, 644 nm, and 663 nm were then measured relative to a blank sample of 85% liquid acetone.

Levels of chlorophyll a and b, total chlorophyll, and carotenoids were determined spectrophotometrically using the following equations (where E stands for absorbance at the indicated wavelength) [17]:

$$\text{Chlorophyll a } (\mu\text{g/mL}) = 10.3 \times E_{663} - 0.98 \times E_{644}$$

$$\text{Chlorophyll b } (\mu\text{g/mL}) = 19.7 \times E_{644} - 3.87 \times E_{663}$$

$$\text{Total chlorophyll} = \text{chlorophyll a} + \text{chlorophyll b}$$

$$\text{Total carotenoids } (\mu\text{g/mL}) = 4.2 \times E_{452.5} - [(0.0264 \times \text{chl a}) + (0.426 \times \text{chl b})]$$

The absorbance of centrifuged, transparent acetone over the range of 380–700 nm was estimated using Spectrophotometer (JASCO Model V-670).

2.2 Detection System:

Laser-induced chlorophyll fluorescence (LICF) values were obtained by exciting the samples with the 2nd harmonic laser pulse (532 nm) of an Nd:YAG laser (Solar Laser System Co.). The pulse width was 7 ns with a repetition rate of 10 Hz. Fluorescence spectra were collected via optical fiber and analyzed using a high resolution spectrograph (Shamrock Model 302 i-B). Signals were detected by an intensified charge-coupled device-ICCD (Andor Model SR-OPT-8002, 300 L/mm grating, average of 16 signals). The correlations between the chlorophyll fluorescence intensity ratio and photosynthetic pigment content was evaluated based on the mean values of three individual experiments.

3. Results

Plants were examined after 30, 50, and 70 days of treatment with either 1, 2, or 3 ml/L (H₂O) of glyphosate. Table 1 summarizes the levels of Chl (a+b), carotenoids (Car), log car, log Chl (a+b), $\beta = F_{\text{max}}/F_{\text{min}} = F_{685}/F_{730}$, and log β , as well as the Chl (a+b)/Car ratio after 30, 50, and 70 days of treatment with various glyphosate concentrations. Data in Table 1 are shown as mean standard error of three independent experiments. Values in parentheses indicate percent decreases (-) or increases (+) relative to the control value.

Table 1: Content of photosynthetic pigment after 30, 50, and 70 days of treatment with different concentrations of glyphosate.

		Glyphosate concentration (ml/l (H ₂ O))	Chl (a+b) 2.3 (µg/mL)	Car 0.3 (µg/mL)	Log Chl (a+b)	Log Car	Chl (a+b)/Car 0.1)	β (0.15)	logβ
After 30 days			65.6	13.59	1.817	1.133	4.779	3.259	0.514
	0 (control)		(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
	1		33.97 (-48.21%)	11.66 (-14.2)	1.531 (- 5.74%)	1.066 (-5.91%)	2.91 (-39.108%)	2.93 (-10.095%)	0.468 (- 8.949%)
	2		23.67 (-63.91%)	8.72 (-5.83%)	1.374 (-4.38%)	0.94 (17.03%)	2.71 (-43.29%)	3.6 (+10.463%)	0.556 (+ 8.17%)
	3		19 (-71.03%)	7.65 (-43.7%)	1.278 (-29.66%)	0.883 (22.06%)	2.48 (-48.106%)	3.74 (+14.759%)	0.5728 (+11.439%)
After 50 days			90.756	24.29256	1.957	1.385	3.73	1.959	0.292
	0 (control)		(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
	1		82.5712 (-9.018%)	23.69 (-2.47%)	1.952 (-0.255%)	1.374 (- 0.794%)	3.77 (+1.07%)	2.087 (+6.533%)	0.3195 (+9.417%)
	2		67.04 (-26.13)	13.7099 (-43.55%)	1.826 (- 6.69%)	1.137 (-17.90%)	4.89 (+ 31.09%)	2.923 (+49.2%)	0.4658 (+ 59.52%)
	3		57.6629 (-36.46)	9.9522 (-59.027%)	1.76 (- 10.066%)	0.997 (-28.01%)	5.78 (+ 54.96%)	2.9(+ 48.03%)	0.462 (+58.219%)
After 70 days			84.4969	25.905	1.926	1.413	3.8344	1.63	0.212
	0 (control)		(100%)	(100%)	(100%)	(100%)	(100%)	(100%)	(100%)
	1		77.7 (-8.02%)	18.226 (-9.64%)	1.89 (- 1.86%)	1.260 (- 0.82%)	4.26 (+11.1%)	1.893 (+ 16.13%)	0.277 (+30.66%)
	2		60.036 (-28.94%)	10.6277 (- 8.97%)	1.778 (- 7.68%)	1.026 (- 7.39%)	5.66 (+47.6%)	2.084 (+27.85%)	0.319 (+50.47%)
	3		63.737 (-24.57%)	11.983 (- 3.74%)	1.804 (- 6.33%)	1.078 (- 23.7%)	5.356 (+39.68%)	2.108 (+29.32%)	0.324 (+52.83%)

N.B. Data are means ± standard error of three independent experiments. Values in parenthesis indicate percent decreases (-) or increases (+) relative to control value.

3.1 Observations after 30 days of treatment

It is clear from Table 1 that the plants exposed to glyphosate 3ml/L (H₂O) contained the lowest levels of Chl (a+b)/car, Chl (a+b), carotenoids (Car), log Car, and log Chl(a+b) and the highest values of β and log β. Plants treated with 3ml/L (H₂O) glyphosate also showed the highest percent decreases in Chl (a+b), car, log Chl (a+b), Chl (a+b)/car, and log Car of -71.03%, -43.7%, -29.66%, -48.106%, and -22.06%, respectively, and the highest changes in β and log β of +14.759% and +11.439%, respectively. It is worth mentioning that β and logβ were -10.095% and -8.949% lower, respectively, than control sample

values. Figure 1 shows the variations in the LICF spectra of the F_{685}/F_{725} ratio (β) for basil leaves resulting from glyphosate treatments of different concentrations and length. The spectra were recorded at various time points (in ms) following laser pulse termination. It is clear that β decreases with increasing glyphosate concentration over the entire time period examined.

The decay of peak intensity of the short wavelength band at an emission wavelength of 677 nm ($F_{\max}$) was recorded immediately from 0 – 750 ms immediately following termination of the laser pulse (i.e. delayed fluorescence) for samples treated with various concentrations of glyphosate (Figure 2). The peak intensities decreased with increasing glyphosate concentration over the entire time period observed. During the recording time used, three distinct phases can be distinguished: 0 to 0.155 ms, 0.155 to 90 ms, and 90 to 750 ms. The largest drop in peak intensity with increasing glyphosate levels occurs during this first recording time range. Figure 3 shows the peak intensity decay of the long wavelength band at 727 nm ($F_{\min}$) over time for samples treated with various glyphosate concentrations. The measurements were recorded immediately after laser pulse termination from 0 to 750 ms.

3.2. Observations after 50 days of treatment

The reduction in Chl (a+b)/car with increasing glyphosate concentration is shown in Table 1. The lowest values of Chl(a+b), Car, log car, and log Chl(a+b) were obtained using glyphosate concentrations of 3 ml/L (H_2O). The highest values for the parameters β and log β were found in samples treated with 2 ml/L (H_2O) glyphosate. Treatment with 3 ml/L glyphosate yielded the highest percent reductions in levels of Chl(a+b), car, log Chl(a+b) of -36.46 %, -59.027%, and -10.066%, respectively. In addition, treatment with 3 ml/L (H_2O) glyphosate caused the highest percent increase in Chl (a+b)/car (54.96%) and the highest percent reduction in log Car (-28.01%). In contrast, treatment with 2 ml/L (H_2O) glyphosate caused the largest percent increases in β (+49.2 %) and log β (+59.52%).

LICF spectra of F_{685}/F_{725} ratio (β) were recorded for basil leaves after 50 days of treatment with various concentrations of glyphosate (Figure 4). Spectra were recorded over the same time period after laser pulse termination as for the 30-day-treatment samples. β values clearly decreased with increasing glyphosate concentration over the entire time period examined. The delayed fluorescence of the shorter wavelength (F_{725}) peak intensity of samples from treatments with different concentrations of glyphosate is shown in Figure 5. The peak intensity decreased with increasing glyphosate concentration. The control sample showed the highest intensity values. Nearly the same trends were observed for the long wavelength band at 677 nm.

3.3. Observations after 70 days of treatment

As shown in Table 1, the lowest amounts of Chl(a+b), Car, log Car, and log Chl (a+b) were found in plants treated with 2 ml/L (H_2O) glyphosate. The highest values for the parameters β and log β were found in basil treated with 3 ml/L (H_2O) glyphosate (Figure 6). The highest percent decrease in Chl(a+b) occurred in basil treated with 2 ml/L (H_2O) glyphosate (-28.94 %) while the highest percent decrease in Car occurred in plants treated with 3 ml/L (H_2O) (-58.97%). The highest percent decrease in log Chl (a+b) -7.68% in plants treated with 2 ml/L (H_2O) glyphosate. The highest percent increase in Chl (a+b)/car +47.6% after treatment with 1 ml/L (H_2O) glyphosate. For log Car, the highest percent decrease (-7.39%) resulted from treatment with 2 ml/L (H_2O) glyphosate. Finally, the highest increase in β (+29.32%) and log β (+55.83%) resulted from treatment with 3 ml/L (H_2O) glyphosate.

4. Discussion

4.1. Plant growth parameters

After 30 days of treatment with 1 ml/l H_2O glyphosate, plant growth parameter of basil plants were improved compared to the control sample, while such parameters worsened after 50 and 70 days of treatment (data not shown). This behavior may emerge from the following processes. Low concentrations of glyphosate significantly stimulated the growth performance of

seedlings after 30 days of treatment, although such effects were unclear after 20 days of treatment. This improvement in growth could have been due to the cellular degradation of glyphosate, an organophosphorus insecticide, ultimately leading to an increase in phosphate content and accelerated plant growth [18]. Glyphosate is absorbed rapidly by plants and translocated to the leaf tissue where most of it is processed.

There have been several reports describing the increased growth of plants and cyanobacteria treated with low doses of dimethoate [12, 19, 20]. Tian et al. [20] found that low concentrations of dimethoate stimulated increased cell density, protein content, Chl content, and alkaline phosphatase activity in *Chlorella vulgaris*. In addition, they found that low doses of glyphosate likely increase cell membrane permeability, causing enhanced nitrite influx to root cells and subsequently to leaf cells and accelerating plant growth performance. Other reports have also observed that organophosphorus insecticides increase membrane fluidity and permeability to ionic and non-ionic compounds [21].

4.2 Photosynthetic pigment content

Pigment content was lower in basil plants treated with 1, 2, or 3 ml/L (H₂O) glyphosate for 30, 50, or 70 days of treatment compared to control plants. The Chl/Car ratio decreased with increasing glyphosate concentration, indicating greater susceptibility of Chl b to glyphosate treatment compared to Chl a. These data also support a protective role for carotenoids during stress. We also observed decreased levels of photosynthetic pigments after 20 and 30 days of treatment for all for all glyphosate concentrations used. This is likely due to decreased Chl biosynthesis via inhibition of d-aminolevulinic acid dehydratase and protochlorophyllide reductase activities and breakdown of pigments and pigment precursors, as reported for other stresses [22, 23]. Unlike plant growth parameters, photosynthetic pigment content continuously decreased with increased treatment time. In addition, this degradation and decline was higher for carotenoids than for Chl (a+b) (Tables 1).

4.3 Laser-induced chlorophyll fluorescence spectra

The fluorescence spectra of basil leaves showed two fluorescence maxima, one in the red region near 685 nm and the other in the far-red region near 725 nm. The intensity of the fluorescence peak near 685 nm displayed greater variation with changes in glyphosate concentration than the 725 nm peak, which showed less sensitivity and variation. Variations in the Chl fluorescence intensity at 685 nm arise from changes in Chl content, which alter the reabsorption of this fluorescence band [2]. In our experiments, after 30 days of treatment, the fluorescence intensity was minimal for basil leaves treated with 0.5 ml/L of glyphosate, while after 30 days of treatment, the Chl fluorescence intensity of basil leaves treated with 1 or 1.5 ml/L of glyphosate was higher than in control plants (Table 1). Additionally, after 50 and 70 days of treatment, the Chl fluorescence intensities of plants treated with all concentrations of glyphosate were higher than the control plants (Table 1). Additionally, the red to far-red fluorescence ratio (F_{685}/F_{730} ; β) was inversely correlated with Chl (a + b) content.

These spectra results indicate that the intensities of red and far-red Chl fluorescence were significantly affected by glyphosate treatment. After 30 days of treatment with 1 ml/L H₂O glyphosate, basil plants exhibited increased photosynthetic pigment content and decreased fluorescence intensity at 685 nm compared to control plants. This decrease in fluorescence intensity around 685 nm region is due to the re-absorption of Chl fluorescence by the Chl absorption band, as Chl absorption and Chl fluorescence emission bands overlap from 680–685 nm. The leaves of the treated plants exhibited increasing fluorescence emission intensity with decreasing pigment content compared to control plants. This increase in fluorescence intensity was observed for plants treated with each glyphosate concentration and for each treatment length. This increase is likely due to decreased re-absorption at 685 nm arising from the reduction in photosynthetic pigment content, as about 90% of the emitted Chl fluorescence at 685 nm is re-absorbed by Chl molecules in green leaves. This re-absorption occurs because of the overlap of F_{685} with the Chl absorption band in the 680 nm region [16, 24]. In addition, at typical room and field temperatures, the Chl fluorescence of PS I is extremely low and can be neglected [25, 26].

The overall Chl fluorescence signal measured from leaves is mixture of signals emitted from different within of the leaf tissue. Since much of the re-absorption of red Chl fluorescence occurs in the deeper layer of cells, most of the red light isn't reabsorbed. In contrast, due to the very low re-absorption by Chl, far red Chl fluorescence emission extends into the deeper

layers of cells. This means that the measured red Chl fluorescence emission is mostly produced by cells on the leaf surface, whereas far red Chl fluorescence is also produced by deeper layers of cells. This creates a nonlinear relationship between red and far red Chl fluorescence levels [27, 28]. Correlations of FIR values and Chl content in basil leaves after 30, 45, and 70 days of glyphosate treatment are shown in Figure. 6 .The red-to-far-red fluorescence intensity ratio (F685/F730) is inversely correlated with total Chl content (Table 1), which clearly explains the concurrent increase in FIR values and decrease in Chl content.

4.4 Fluorescence damping kinetics on the millisecond timescale

The decline in the Chl fluorescence is a function of the potential photosynthetic capacity of plant leaves. The increased intensity as indicated by values of F680, F730 and, β values for plants treated with 1, 2, and 3 ml/L (H₂O) of glyphosate for 30, 50, and 70 days of treatment is correlated to increased photosynthetic activity. Such increases are likely related to an increase in potential photosynthetic activity and capacity of plant leaves and has thus has been dubbed a “plant vitality index” [12]. The increase in F680, F730, and β value was lower with increasing concentration of in glyphosate, which may be due to a decrease in photosynthetic activity. Such a decrease in F680, F730, and β value at higher concentrations of glyphosate is correlated with a decrease in photosynthetic pigment content as well as with damage caused to the photosynthetic apparatus[19, 29]. As part of Chl fluorescence damping kinetics, a decline in F680, F730, and β values parallels the oxygen evolution [12]. In addition, glyphosate influences membrane integrity as it is a dimethoate compound [6]. This overall suggests that the electron transport activity of photosynthetic electron transport chain is reduced and net photosynthetic output of plant leaves reduced in leaves treated with glyphosate. Our results showed that β and fluorescence peak intensity decreased with increases in glyphosate concentration after 30 and 50 days of treatment, especially for measurements taken from 100 ms to 800 ms after laser pulsing (Figure. 1, 4). This is evidence of the effect of glyphosate on electron transport in in PS II, especially between S1, S2, S3, and S4, and on photosynthetic water oxidation.

5. Conclusions

The present study involved planned time course experiments designed to assess the effects of the pesticide glyphosate on the photosynthetic pigment content, Chl fluorescence, and photosynthetic activity of basil plants. The results of this investigation show that the intensity of Chl fluorescence and fluorescence intensity ratio F685/F730 are greatly dependent on the Chl content of the leaves. The decline in Chl content due to glyphosate stress for short- and long-term durations of treatment increased the fluorescence intensity in the 685 nm region and FIR. The decrease in values of F680, F730, and β value with increasing concentrations of glyphosate and treatment duration indicated a reduction in photosynthetic CO₂ fixation rate. Thus, our result confirm the usefulness of Chl fluorescence measurements in the nondestructive and remote evaluation of plant health, photosynthetic pigment content, and net photosynthetic activity under glyphosate stress in basil plants. Glyphosate is a widely-used insecticide, so this investigation is helpful in determining the optimal dose of glyphosate for agricultural practices.

Declarations

Consent for publication

Not applicable.

Availability of data and material

The data presented in this study are available on request from the corresponding author.

Competing interests

The authors declare no conflict of interest.

Authors' contributions

Conceptualization, W.T. and A.S.A.; methodology, W.T. and A.S.A.; validation, W.T. and A.S.A.; formal analysis, W.T., A.S.A. and H.M.G.; investigation, W.T., A.S.A. and H.M.G.; resources, W.T., A.S.A.; data curation, W.T., A.S.A. and H.M.G.; writing—original draft preparation, W.T.; writing—review and editing, W.T., A.S.A. and H.M.G.; visualization, W.T., A.S.A.; supervision, A.S.A.; project administration, A.S.A.; funding acquisition, W.T., A.S.A. All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1

Variations in the LICF spectra of F685/F725 ratio (β) of basil leaves after 30 days of treatment with time in millisecond.

Figure 2

Variation of the maximum intensity of the peak F677 with times in millisecond.

Figure 3

Variation of peak intensity for F727 line over microseconds regimes.

Figure 4

Variations LICF spectra of F685/F725 ratio (β) after 50 days of treatment.

Figure 5

Variation of peak intensity at F725 with time.

Figure 6

The relationship between $\log \beta$ with the concentration of glyphosate (ml/l(H₂O))