Detection of the onset of glyphosate-induced soybean plant injury through chlorophyll fluorescence signal extraction and measurement

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Abstract. In this study, chlorophyll fluorescence (ChlF) was used to detect the onset of soybean plant injury from treatment of glyphosate, the most widely used herbicide. Thirty-six pots of nonglyphosate-resistant soybean were randomly divided into three groups and treated with different doses of glyphosate solutions. The three treatment groups were control (CTRL) group (with no glyphosate treatment), 0.25X group (treated with $0.217\ \mathrm{kg \cdot ae/ha}$ solution of glyphosate), and 0.5X group (treated with $0.433\ \mathrm{kg \cdot ae/ha}$ solution of glyphosate). Three kinds of fluorescence measurements, steady-state fluorescence spectra, Kautsky effect parameters, and ChlF-related spectral indices were extracted and generated from the measurements in the glyphosate treatment experiment. The mean values of these fluorescence measurements for each of the CTRL group, the 0.25X group, and the 0.5X group were calculated. Glyphosate-induced leaf injury was then analyzed by examining the separability of these mean values at 6, 24, 48, and 72 hours after the treatment (HAT). Results indicate that the peak position of far-red ChlF shows an obvious blue shift for glyphosate-treated soybean, and peak values of steady-state fluorescence spectra for the three groups can be significantly distinguished from each other at 48 HAT and later. Four Kautsky effect parameters, Fv, Fv/Fm, Area, and PI, are parameters sensitive to glyphosate treatment, showing some differences between the CTRL group and treated groups at 24 HAT, and significant differences among the three groups at and beyond 48 HAT. Moreover, ChlF-related spectral indices, $R_{683}^2/(R_{675} \cdot R_{690})$ and R_{690}/R_{655}, are also shown to be useful in detection of the glyphosate injury, though they are less effective than the steady-state fluorescence spectra and the Kautsky effect parameters. Based on the presented results, it can be concluded that glyphosate-induced soybean injury can be detected in a timely manner by the ChlF measurements, and this method has the potential to be further developed into practical use. © *2015 Society of Photo-Optical Instrumentation Engineers (SPIE)* [DOI: 10.1117/1.JRS.9.097098]

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1 Introduction

For many years, glyphosate has been widely applied to genetically modified glyphosate-resistant (GR) crops as an herbicide aiming to control weeds.[1] However, as glyphosate is

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a nonselective, systemic herbicide highly toxic to sensitive species, it may cause severe damage if it drifts onto non-GR crops, and farmers could, therefore, incur significant losses.[2] This problem has become more severe recently due to the increased use of glyphosate, which makes the early detection of crop injury caused by glyphosate drift a crucial problem for field managers.[3]

Remote sensing is a technique that has been introduced as a noninvasive and rapid method for detecting glyphosate injury in the past few years.[4] Some studies have been reported which analyze the effectiveness of employing reflectance-based spectral indices and transformed spectral features in detecting glyphosate injury.[1,5] For example, in a previous study, a total of 14 vegetation indices were calculated from the leaf reflectance spectra to detect the glyphosate-induced soybean injury.[5] In the experiment, the soybean plants were randomly divided into three groups and treated with different dosages of glyphosate solutions. It was found that the three treatment groups could be significantly distinguished from each other by normalized difference vegetation index (NDVI) and ratio vegetation index (RVI) at 72 hours after the treatment (HAT). However, the reflectance-based methods have suffered from two main drawbacks. First, the traditionally used spectral indices and features derived from reflectance data, such as the NDVI and RVI, are sensitive to low chlorophyll contents but tend to saturate at higher chlorophyll levels.[6] Second, leaf reflectance spectra can reflect foliar optical properties, but they give no insight into the biophysical status of plants.[7] Leaf chlorophyll fluorescence (ChlF), compared against the reflectance data, is closely associated with the photosynthesis process,[8] and could be employed as a more direct proxy of the physiological state of plants.[9] Moreover, ChlF is emitted by the photosynthetic apparatus itself and is still sensitive to plant stress conditions when chlorophyll content is high,[10] and would, therefore, not be affected by the saturation effect.

Both the Kautsky effect parameters[1] and ChlF-related spectral indices[11] can be found in studies that were used as proxies of ChlF to evaluate the stress conditions of plants. Kautsky and Hirsch[12] found that the fluorescence intensity of the dark adapted leaf shows a rapid polyphasic rise upon being illuminated, which is known as the Kautsky effect. The parameters derived from the Kautsky effect curve, such as the original fluorescence (Fo) and the maximum fluorescence (Fm), are shown to be closely related to leaf vigor.[13,14] In addition, ChlF-related spectral indices can reflect the effect of ChlF on the leaf reflectance spectra within the spectral region of ChlF emission.[9] These indices have been shown to be related to the ChlF intensity,[15] and could be used to monitor crop growth conditions.

As a previsual indicator highly sensitive to plant stress conditions, ChlF has been successfully used for monitoring stress induced by glyphosate. In a glyphosate treatment experiment, Huang et al. measured the Kautsky effect parameters with a portable chlorophyll fluorimeter for glyphosate treated and untreated soybean plants, and found that some parameters [e.g., performance index (PI) and Area] could identify the herbicide effect within 1 day after treatment.[1] However, only the Kautsky effect parameters were measured in this study. The effectiveness of using other ChlF measurements, such as the steady-state fluorescence spectra and ChlF-related indices, for detecting the soybean injury from herbicide treatment needs to be further investigated and compared.

In this study, we conducted a more thorough study on the effectiveness of using ChlF for detecting the onset of glyphosate-induced soybean stress with the objectives:

1. Study three kinds of fluorescence measurements, steady-state fluorescence spectra, Kautsky effect parameters, and ChlF-related indices in a glyphosate treatment experiment for high-dose, low-dose, and no glyphosate-treated soybean plants;
2. Analyze and compare these fluorescence measurements statistically to investigate if there are significant differences among different treatment levels at different time periods after the glyphosate treatment.

The rest of this paper is organized as follows: in Sec. 2, we introduce the experimental design and the methods to acquire and extract the fluorescence signal; in Sec. 3, the results for detecting the glyphosate-induced soybean injury are presented, as well as some discussions on the potential of this study; Sec. 4 gives the concluding remarks.

2 Experiments and Methods

2.1 *Experimental Design*

One type of commercial non-GR soybean (cultivar SO80120LL) was used in the experiment to acquire ChlF measurements during February 4 to 7, 2013. A total of 36 pots of soybean were used for the experiment, with one soybean plant in each pot. The complete randomized design was used in the experiment, with all pots being randomly divided into three treatments: 12 pots were sprayed with 0.433 kg · ae/ha solution of glyphosate (0.5X group; $X = 0.866$ kg · ae/ha, which is the label rate of the commercial glyphosate we used; ae stands for acid equivalent); another 12 pots were sprayed with half of the 0.5X dose (0.25X group); the remaining 12 pots were used as controls with no glyphosate treatment (CTRL group). The glyphosate solutions were prepared using a commercial formulation of the potassium salt of glyphosate (Roundup WeatherMax, Monsanto Agricultural Co., St. Louis, Missouri), and applied using a CO_2-pressurized backpack sprayer that delivered 140 L/ha of spray solution at 193 kPa. All plants were raised under controlled conditions in a greenhouse at the USDA-ARS Crop Production Systems Research Unit, Stoneville, Mississippi. Three plants from each group were used for measurement at 6, 24, 48, and 72 HAT, respectively.

2.2 *Measurement of Leaf Steady-State Fluorescence Spectra*

Leaf steady-state fluorescence spectra were acquired by using an analytica spectra devices (ASD) integrating sphere apparatus coupled with an ASD FieldSpec 3 Hi-Res spectroradiometer (ASD Inc., Boulder, Colorado) and a removable filter [Fig. 1(a)]. Similar to the procedure used by Zarco-Tejada et al.,[11] a long-pass red filter (10LWF-700-B, Newport Co., Irvine, California) is placed in front of the spare lamp with irradiances shorter than 700 nm being cut-off [Fig. 1(b)]. The reflectance of the leaf sample was measured twice, once with the filter on and once with the filter off the integrating sphere. Since the spectral range that can excite the ChlF is from 400 to 700 nm, measurements with the long-pass red filter in front of the light source should not include the ChlF signal. With the filter off, the measured reflectance spectrum contains the contribution of both the reflected radiance and ChlF.

The reflectance spectra were measured following the procedure described in the ASD integrating sphere manual[16] in which three series of measurements from 400 to 2500 nm, with a spectral resolution of 3 nm and spectral sampling interval of 1nm, are required: sample measurement (I_s), stray light measurement (I_d), and reference standard measurement (I_r). These spectra were collected in raw digital number (DN) mode. An integration time of 544 ms was used for all the measurements. With the known reflectance of the reference standard R_r, the reflectance of the sample for a given center wavelength and spectral bandpass R_s is calculated as follows:

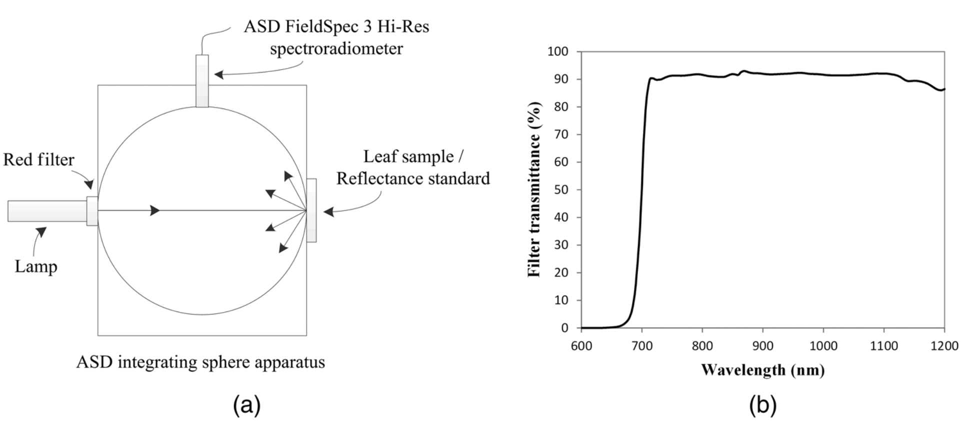

Fig. 1 (a) Sketch of the ASD integrating sphere apparatus used for the measurement of leaf fluorescence spectra. (b) The transmittance of the red filter within the spectral region of 600-1200 nm.

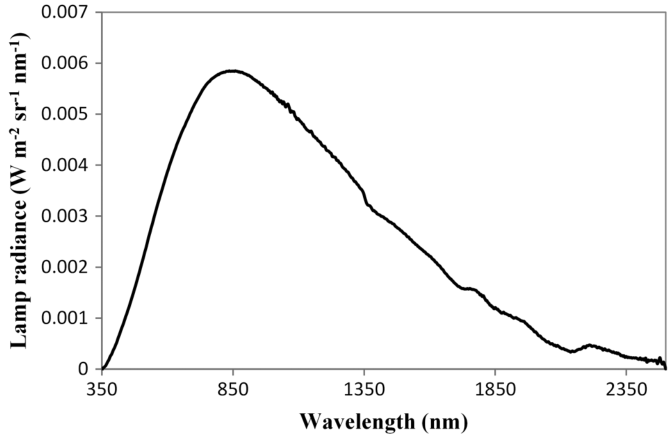

Fig. 2 Spectral radiance of the lamp illuminating leaf samples and the Reference Standard in the experiment.

$$R_s = \frac{(I_s - I_d)R_r}{I_r - I_d}. \tag{1}$$

With the filter on and off, the reflectance spectra were denoted as R_s^{on} and R_s^{off}, respectively. The R_s^{off} is the apparent reflectance, which contains the contribution of ChlF radiance (F). The R_s^{on} is the actual reflectance and does not contain the contribution of F. The relationship among R_s^{on}, R_s^{off}, and F can be expressed as

$$R_s^{\text{off}} = \frac{L_{\text{lamp}} \cdot R_s^{\text{on}} + F}{L_{\text{lamp}}}, \tag{2}$$

where L_{lamp} is the radiance of the light source as shown in Fig. 2. Then, the ChlF radiance F was calculated using the following equation derived from Eq. (2):

$$F = (R_s^{\text{off}} - R_s^{\text{on}}) \cdot L_{\text{lamp}}. \tag{3}$$

The ChlF radiance spectrum was calculated for each leaf within the spectral region of 730 to 850 nm, where the ChlF is emitted and the transmittance of the filter is higher than 90% [Fig. 1(b)]. As a result, only the far-red peak of the ChlF spectra (located around 740 nm) can be observed. The mean spectrum was then averaged from all leaf samples in each treatment group at each time period after the treatment. The spectra of different treatment groups were compared to study their responses to glyphosate treatment.

2.3 *Measurement of Leaf Kautsky Effect Parameters*

A Handy PEA chlorophyll fluorimeter (Hansatech Instruments Ltd., Norfolk, United Kingdom) was used to acquire leaf Kautsky effect parameters. The measurements were conducted under guidance of the operation manual of the Handy PEA chlorophyll fluorimeter.[17] The leaf sample was first dark adapted for 15 min, which was sufficient to ensure that the sample was fully dark adapted. Then, the leaf sample was illuminated with a light intensity of 3000 μmol m^{-1} s^{-1} to ensure that the sample was fully saturated during the measurement. Starting from the illumination, the fluorimeter recorded the fluorescence intensity for 2 s by sampling data points in units of bits. Each leaf sample was measured three times by repeating the routine described above.

Kautsky effect parameters, Fo, Fm, Fv, Fv/Fm, Tfm, and Area, were recorded by the fluorimeter in each measurement. Among these, Fo represents the original fluorescence, which is the initial level of fluorescence upon illumination. Fm represents the maximum fluorescence, which is the peak fluorescence level achieved after illumination. Fv is variable fluorescence, which is calculated by subtracting the Fo value from the Fm value. Fv/Fm is the ratio of Fv to Fm. Tfm indicates the time at which the Fm value was reached. Area is the area above

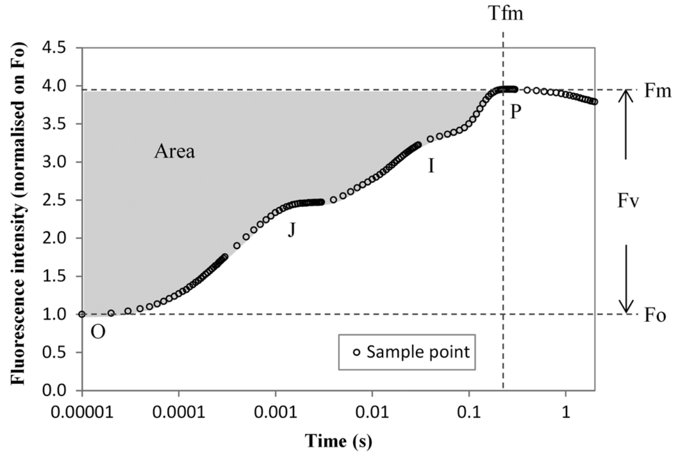

Fig. 3 A typical Kautsky effect curve recorded by the Handy PEA chlorophyll fluorimeter. Sample points are plotted on a logarithmic time scale. Four phases in the polyphasic rise are denoted with letters O, J, I, and P. Fo, Fm, Fv, Tfm, and Area, which are meaningful fluorescence parameters derived from these sample points, are also illustrated.

the Kautsky induction curve from the time of illumination to the time Fm was achieved. These parameters are illustrated in Fig. 3. Another parameter, PI, which is an indicator of leaf vitality, was also calculated according to the method given by Strasser et al.[13,14]

2.4 *Calculation of Chlorophyll Fluorescence-Related Spectral Indices from Leaf Reflectance Spectra*

Four ChlF-related spectral indices were calculated from the apparent reflectance spectra (i.e., the R_s^{off} measured without the filter). These leaf-level indices were proposed by Zarco-Tejada et al.[11] and calculated as $R_{683}^2/(R_{675} \cdot R_{690})$, R_{750}/R_{800}, R_{685}/R_{655}, and R_{690}/R_{655}. The R_{683}, R_{675}, R_{690}, R_{750}, R_{800}, R_{685}, and R_{655} represent the apparent reflectance values at 683, 675, 690, 750, 800, 685, and 655 nm, respectively. These indices could be used to extract ChlF information from leaf apparent reflectance spectra. Their changes are correlated with the ChlF effect and, therefore, can potentially represent the physiological status and stress conditions of soybean leaves.

2.5 *Statistical Analysis*

Means of the steady-state fluorescence spectra, the Kautsky effect parameters, and the ChlF-related spectral indices were calculated for the CTRL, 0.25X, and 0.5X groups at 6, 24, 48, and 72 HAT. Duncan's multiple range tests with a p-value of 0.05 (0.05 confidence probability) were applied to differentiate these mean values. SPSS 19 Statistics (SPSS Inc., Chicago, Illinois) was used for the analysis.

3 Results and Discussions

3.1 *Variations in Leaf Steady-State Fluorescence Spectra*

Figure 4 shows the variations in leaf steady-state fluorescence spectra at each time period after the glyphosate treatment. The spectra of the CTRL, 0.25X, and 0.5X groups were plotted. It can be seen that the spectra of the CTRL group were relatively stable after the treatment, with the peak value remaining constant at around 2.1 $W \cdot m^{-2} \cdot sr^{-1} \cdot nm^{-1}$. The ChlF of the treated groups, however, showed an increasing trend with time. At 6 HAT, the difference among the three groups was not very large. At 24 HAT, the magnitude of the peak ChlF for the 0.25X group became the largest, whereas that for the 0.5X group also showed a slight increase. The treatment effect on the three groups became evident at 48 HAT, with the higher dosage

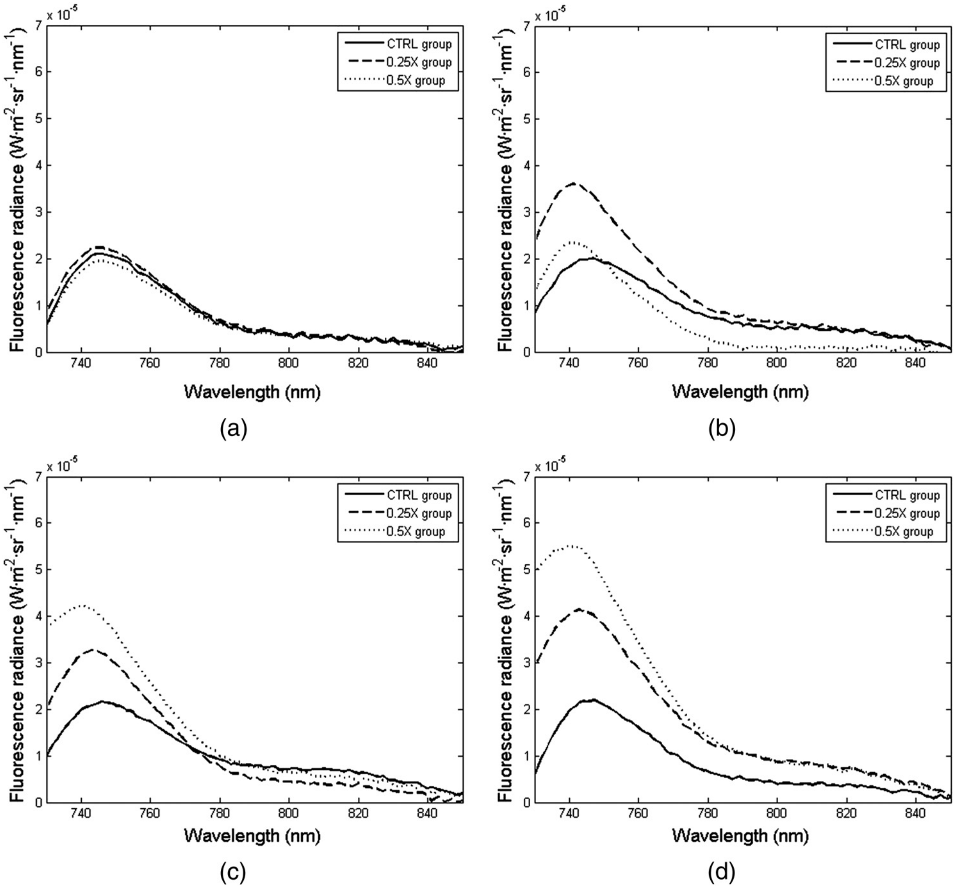

Fig. 4 Fluorescence spectra for all groups after the glyphosate treatment. (a) 6 hours after treatment (HAT); (b) 24 HAT; (c) 48 HAT; (d) 72 HAT. Each spectrum was averaged from three steady-state fluorescence spectra in the same group.

treatment group (0.5X group) showing a higher ChlF value. At 72 HAT, the differences of ChlF values of the three groups became more significant.

Table 1 summarizes the ChlF radiance at the position of the ChlF peaks for different treatment groups at each time period. The separation analysis of these values was conducted using Duncan's multiple range test. It can be seen that the three groups can be totally separated at and beyond 48 HAT, with significant differences between each two groups. This indicates that the

Table 1 Mean chlorophyll fluorescence (ChlF) values at the wavelength positions of the ChlF peaks for the three treatment groups (CTRL group with no glyphosate treatment; 0.25X group treated with 0.217 kg · ae/ha solution of glyphosate; 0.5X group treated with 0.433 kg · ae/ha solution of glyphosate) at each time period [6, 24, 48, and 72 h after treatment (HAT)]. Each value was averaged from three ChlF spectra of the same group. These values are in units of $W \cdot m^{-2} \cdot sr^{-1} \cdot nm^{-1}$. The separation results are based on Duncan's multiple range test. Means with the same letter (a, b, or c) in each row are not significantly different at the 0.05 level of probability.

Time	CTRL group	0.25X group	0.5X group
6 HAT	2.11×10^{-5a}	2.23×10^{-5a}	1.95×10^{-5a}
24 HAT	2.01×10^{-5b}	3.52×10^{-5a}	2.29×10^{-5b}
48 HAT	2.16×10^{-5c}	3.27×10^{-5b}	4.21×10^{-5a}
72 HAT	2.19×10^{-5c}	4.14×10^{-5b}	5.50×10^{-5a}

peak value of the steady-state fluorescence spectra is a good indicator of the glyphosate-induced soybean stress.

In previous studies it was found that, without the occurrence of the nonphotochemical quenching, the ChlF was shown to be inversely related to the photosynthetic rates.[7,18,19] Because the photosynthetic rates of the glyphosate-treated plants would decrease as a result of the direct damage of glyphosate to chlorophyll,[20–22] an increase of the leaf steady-state fluorescence was observed in our study for the glyphosate-treated soybean leaves, as shown in Fig. 4. A similar phenomenon of increase in ChlF was also observed for the palm and grape plants treated by herbicide dichlorophenyldimethylurea,[23] and for the corn and soybean plants treated by herbicide bromacil.[24]

The most obvious features of the ChlF spectra are the far-red peaks around 740 nm. These peaks are attributed to the ChlF emission of the photosystem I (PS I).[7] A shift of the wavelength, corresponding to the far-red ChlF peak, to the shorter band (blue shift) was observed for the glyphosate-treated groups, especially for the 0.5X group, as shown in Table 2. It can be seen that the peak positions for the CTRL group stabilized at 746 to 747 nm for all four time periods. But for the glyphosate-treated groups, the blue shift of the ChlF peak could be seen. The trend of the blue shift was more and more pronounced with time, and more obvious for the 0.5X group than the 0.25X group. From 6 to 72 HAT, the shift of the ChlF peak was 1 nm for the 0.25X group and 5 nm for the 0.5X group.

A blue shift of the far-red ChlF peak position (740 nm) was reported for the nitrogen-, phosphorus-, and potassium-deficient sunflowers by Subhash and Mohanan.[25] A similar phenomenon was observed in our study, as shown in Table 2. The trend of the blue shift is consistent with time and more pronounced for the high dosage treated group (0.5X group). The decrease of chlorophyll content with time, which is confirmed by our measured data for the glyphosate-treated soybean used in this study,[26] could induce the shift of a few nanometers for the 740-nm ChlF peak.[27] But the decrease of chlorophyll content was supposed to only have a minor effect on the shift for the far-red ChlF band.[25] The glyphosate-induced stress could be another reason to cause the shift. But to fully explain the underlying mechanism of its shifts need thorough investigations and more experimental studies. Besides, we should note that the blue shift observed for the 0.25X group in this study was 1 nm from 6 to 72 HAT. However, the relatively coarse spectral resolution of the ASD spectroradiometer (3 nm) used in this study should not be sensitive enough to discern this shift. Therefore, it is suggested that instruments with higher spectral resolutions (e.g., 1 nm) should be used in future studies.

3.2 *Variations in Leaf Kautsky Effect Parameters*

Mean values of the Kautsky effect parameters of the CTRL, 0.25X, and 0.5X groups at 6, 24, 48, and 72 HAT are shown in Table 3. It can be seen that the groups treated with higher rates of glyphosate solution tended to have higher Fo values and lower Fm values after 6 HAT, which resulted in smaller Fv and Fv/Fm values. Meanwhile, another two parameters, Area and PI, tended to increase with time. Results of the Duncan's multiple range test indicated that the

Table 2 Peak positions of the far-red chlorophyll fluorescence (ChlF) for the three treatment groups (CTRL group with no glyphosate treatment; 0.25X group treated with 0.217 kg · ae/ha solution of glyphosate; 0.5X group treated with 0.433 kg · ae/ha solution of glyphosate) at each time period [6, 24, 48, and 72 hours after treatment (HAT)]. Each value was averaged from three ChlF spectra of the same group.

Time (HAT)	CTRL group (nm)	0.25X group (nm)	0.5X group (nm)
6	746	744	745
24	747	744	744
48	746	743	741
72	747	743	740

Table 3 The Kautsky effect parameters of the three treatment groups (CTRL group with no glyphosate treatment; 0.25X group treated with 0.217 kg · ae/ha solution of glyphosate; 0.5X group treated with 0.433 kg · ae/ha solution of glyphosate) at each time period after treatment [6, 24, 48, and 72 hours after treatment (HAT)]. Each value is a mean of three values measured for the same treatment group. The separation results are based on the Duncan's multiple range test. Means with the same letter (a, b, or c) in each row are not significantly different at the 0.05 level of probability.

Group	Fo (bits)	Fm (bits)	Fv (bits)	Fv/Fm (–)	Tfm (ms)	Area (bits·ms)	Performance index (–)
6 HAT							
CTRL	805.8^{a}	2340.3^{a}	1534.6^{a}	0.653^{a}	284.4^{a}	29663^{a}	0.254^{a}
0.25X	844.9^{a}	2692.8^{a}	1847.9^{a}	0.684^{a}	288.9^{a}	38987^{a}	0.282^{a}
0.5X	799.4^{a}	2368.1^{a}	1568.7^{a}	0.662^{a}	237.8^{a}	26004^{a}	0.221^{a}
24 HAT							
CTRL	747.4^{a}	3189.2^{a}	2441.8^{a}	0.766^{a}	542.2^{a}	82859^{a}	1.221^{a}
0.25X	762.8^{a}	2789.0^{a}	$2026.2^{a,b}$	0.716^{b}	243.5^{a}	37627^{b}	0.517^{b}
0.5X	821.8^{a}	2690.4^{a}	1868.7^{b}	0.684^{b}	303.3^{a}	33432^{b}	0.347^{b}
48 HAT							
CTRL	778.9^{b}	3678.7^{a}	2899.8^{a}	0.788^{a}	488.9^{a}	84257^{a}	1.647^{a}
0.25X	810.7^{b}	3300.1^{b}	2489.4^{b}	0.752^{b}	298.9^{a}	51007^{b}	0.813^{b}
0.5X	998.3^{a}	3267.1^{b}	2268.8^{c}	0.690^{c}	374.4^{a}	37965^{c}	0.350^{c}
72 HAT							
CTRL	841.9^{c}	3720.0^{a}	2878.1^{a}	0.774^{a}	600.0^{a}	90337^{a}	1.998^{a}
0.25X	981.2^{b}	3594.8^{b}	2613.6^{b}	0.725^{b}	353.3^{a}	62302^{b}	0.985^{b}
0.5X	1096.2^{a}	3404.3^{b}	2308.1^{c}	0.678^{c}	487.8^{a}	48013^{c}	0.602^{c}

Fo and Fm of the three groups showed some separability at 48 HAT, and at 72 HAT could be totally distinguished from each other. Fv and Fv/Fm of the CTRL group could be distinguished from the 0.5X group at 24 HAT. At and beyond 48 HAT, the three groups could be totally distinguished with significant differences between each other. Moreover, Area and PI were also shown to be good indicators for the detection of the glyphosate-induced soybean stress, with the CTRL group being significantly distinguished from the other two groups at 24 HAT. Similarly, the three groups could be totally distinguished by Area and PI at and beyond 48 HAT. Tfm was found to be a relatively insensitive parameter to glyphosate treatment, showing no useful information for the separation of the treatment groups from 6 to 72 HAT.

Among all the Kautsky effect parameters, Fv/Fm can be used to estimate the maximal photochemical efficiency of Photosystem II and is, therefore, considered to be a sensitive indicator of plant photosynthetic performance.[17] In our study, from 24 HAT on, the Fv/Fm values of the 0.25X and 0.5X groups were significantly lower than that of the CTRL group (Table 3), which indicates a decrease of the photosynthetic rate of the glyphosate-treated soybean leaves.

3.3 *Variations in Chlorophyll Fluorescence-Related Spectral Indices*

Four ChlF-related spectral indices calculated from leaf apparent reflectance spectra were analyzed using the Duncan's multiple range test, as shown in Table 4. It can be seen that at and before 24 HAT, all indices were not significantly different among the three treatment groups. At 48 HAT and later, $R_{683}^2/(R_{675} \cdot R_{690})$ and R_{690}/R_{655} showed some useful information for separating the three groups. With $R_{683}^2/(R_{675} \cdot R_{690})$, the CTRL group could be significantly

Table 4 Chlorophyll fluorescence (ChlF)-related spectral indices of the three treatment groups (CTRL group with no glyphosate treatment; 0.25X group treated with 0.217 kg · ae/ha solution of glyphosate; 0.5X group treated with 0.433 kg · ae/ha solution of glyphosate) at each time period after treatment [6, 24, 48, and 72 hours after treatment (HAT)]. Each value is a mean of three values for the same treatment group. The separation results are based on the Duncan's multiple range test. Means with the same letter (a, b, or c) in each row are not significantly different at the 0.05 level of probability.

Group	$R_{683}^2/(R_{675} \cdot R_{690})$	R_{750}/R_{800}	R_{685}/R_{655}	R_{690}/R_{655}
6 HAT				
CTRL	0.86^a	0.99^a	0.96^a	1.13^a
0.25X	0.87^a	0.99^a	0.97^a	1.12^a
0.5X	0.82^a	0.99^a	0.94^a	1.13^a
24 HAT				
CTRL	0.83^a	0.98^a	0.96^a	1.11^a
0.25X	0.81^a	0.99^a	0.95^a	1.17^a
0.5X	0.87^a	0.99^a	0.93^a	1.16^a
48 HAT				
CTRL	0.96^b	0.98^a	1.03^a	1.12^b
0.25X	$0.84^{a,b}$	0.99^a	0.99^a	$1.19^{a,b}$
0.5X	0.79^a	0.99^a	0.97^a	1.23^a
72 HAT				
CTRL	0.95^b	0.98^a	1.06^a	1.13^b
0.25X	$0.86^{a,b}$	0.99^a	1.03^a	1.18^b
0.5X	0.81^a	0.99^a	1.04^a	1.29^a

distinguished from the 0.5X group at 48 and 72 HAT, whereas the 0.25X group was neither distinguishable from the CTRL nor from the 0.5X group. With R_{690}/R_{655}, the CTRL and 0.5X groups showed significant differences 48 HAT, and at 72 HAT the 0.5X group could be distinguished from the other groups. However, the other two spectral indices, R_{750}/R_{800} and R_{685}/R_{655}, did not provide useful information for the separation. The results indicate that, compared with R_{750}/R_{800} and R_{685}/R_{655}, $R_{683}^2/(R_{675} \cdot R_{690})$ and R_{690}/R_{655} are more informative in detecting glyphosate-induced soybean injury.

The leaf apparent reflectance spectrum is composed of both the contributions of the reflected radiation and the ChlF signal. Therefore, variations in the ChlF signal will affect the shape of the apparent reflectance spectrum, and subsequently induce variations in the ChlF-related spectral indices. However, because the contribution of the ChlF signal to the total measured radiance is relatively small (lower than 3% in the experiment conducted by Zarco-Tejada et al.[11]), the ChlF-related spectral indices are not so sensitive. Therefore, in our study, they were shown to be less effective than the steady-state fluorescence spectra and the Kautsky effect parameters in detecting the glyphosate-induced soybean injury.

3.4 *Potential of Using Chlorophyll Fluorescence to Detect the Glyphosate-Induced Soybean Injury*

It is well accepted that the physiological status of plants could be affected by environmentally induced stress factors.[28–30] Compared with leaf reflectance data, ChlF is emitted by the photosynthesis apparatus itself and can be employed as a more direct way of detecting the onset of plant stress. Results presented in this study indicate that ChlF is a good indicator for the early detection of soybean injury from glyphosate. Blue shifts of the ChlF peaks occur for the glyphosate-treated groups, with different extents for different doses and time periods. Besides, compared with ChlF-related indices, the peak values of the leaf steady-state fluorescence spectra and most of the Kautsky effect parameters are more effective in separating the three treatment groups, with significant differences detectable within 48 HAT, which outperformed the results achieved by using the traditional vegetation indices as reported by Huang et al.[1] and Zhao et al.[5] More work needs to be done to further investigate the potential of this method. In future studies, it is suggested that spectroradiometers of higher spectral resolution, more measurement time intervals, more treatment levels, and more crop species and numbers of samples should be used. In this preliminary study, the experiment was conducted in a greenhouse at the leaf level under controlled conditions to eliminate the influence of out-door uncertainties. In order to develop this method for field use, canopy-level experiments should be conducted to further examine the effectiveness of ChlF in detecting glyphosate-induced soybean stress under natural conditions. At the canopy level, crop architecture and the contribution of soil background contribute a lot to the spectral features,[31] which may confound the detection of glyphosate injury.

4 Conclusion

This work analyzed the feasibility of using ChlF to detect the onset of glyphosate-induced soybean stress. Results indicate that the steady-state fluorescence spectra and the Kautsky effect parameters are good indicators of glyphosate injury, and the ChlF-related spectral indices can provide useful information as well. Based on the presented results, it can be concluded that the glyphosate-induced soybean injury can be detected in a timely manner by the ChlF measurements, and this method has the potential to be further developed for practical use. Future research studies should be undertaken comparing this technique to others for detecting soybean injury due to glyphosate drift.

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References

1. Y. Huang et al., "Early detection of soybean plant injury from glyphosate by measuring chlorophyll reflectance and fluorescence," *J. Agric. Sci.* **4**, 117–124 (2012).
2. H. Yao et al., "Using vegetation index and modified derivative for early detection of soybean plant injury from glyphosate," *Comput. Electron. Agric.* **89**, 145–157 (2012).
3. W. Ding et al., "Biological response of soybean and cotton to aerial glyphosate drift," *J. Crop Improv.* **25**, 291–302 (2011).
4. Y. Huang et al., "Airborne remote sensing assessment of the damage to cotton caused by spray drift from aerially applied glyphosate through spray deposition measurements," *Biosyst. Eng.* **107**, 212–220 (2010).
5. F. Zhao et al., "Early detection of crop injury from glyphosate on soybean and cotton using plant leaf hyperspectral data," *Remote Sens. Basel* **6**, 1538–1563 (2014).
6. S. W. Maier, K. P. Günther, and M. Stellmes, "Sun-induced fluorescence: a new tool for precision farming," in *Digital Imaging and Spectral Techniques: Applications to Precision*

Agriculture and Crop Physiology, T. VanToai et al., Eds., pp. 209–222, American Society of Agronomy, Madison (2003).
7. J. Miller et al., "Development of a vegetation fluorescence canopy model," ESTEC Contract 16365/02/NL/FF Final Report (2005). http://www.ias.csic.es/fluormod/documents/FluorMOD_Final_Report_April_13_2005.pdf.
8. C. Van der Tol, W. Verhoef, and A. Rosema, "A model for chlorophyll fluorescence and photosynthesis at leaf scale," *Agric. Forest Meteorol.* **149**, 96–105 (2009).
9. M. Meroni et al., "Remote sensing of solar-induced chlorophyll fluorescence: review of methods and applications," *Remote Sens. Environ.* **113**, 2037–2051 (2009).
10. U. Rascher et al., "Sensing of photosynthetic activity of crops," in *Precision Crop Protection - the Challenge and Use of Heterogeneity*, E. C. Oerke et al., Ed., pp. 87–99, Springer, The Netherlands (2010).
11. P. J. Zarco-Tejada et al., "Chlorophyll fluorescence effects on vegetation apparent reflectance: I. Leaf-level measurements and model simulation," *Remote Sens. Environ.* **74**, 582–595 (2000).
12. H. Kautsky and A. Hirsch, "Neue Versuche zur Kohlensäureassimilation," *Naturwissenschaften* **19**, 964 (1931).
13. R. J. Strasser, A. Srivastava, and M. Tsimilli-Michael, "The fluorescence transient as a tool to characterize and screen photosynthetic samples," in *Probing Photosynthesis: Mechanisms, Regulation and Adaptation*, pp. 445–483, Taylor and Francis Press, London (2000).
14. R. J. Strasser, M. Tsimilli-Michael, and A. Srivastava, *Analysis of the Chlorophyll a Fluorescence Transient*, pp. 1–47, Springer, The Netherlands (2003).
15. S. Z. Dobrowski et al., "Simple reflectance indices track heat and water stress-induced changes in steady-state chlorophyll fluorescence at the canopy scale," *Remote Sens. Environ.* **97**, 403–414 (2005).
16. *Integrating Sphere User Manual*, ASD Document 600660 Rev. B, ASD Inc.: Boulder, Colorado (2008).
17. *Operations Manual: Setup, Installation & Maintenance*, Handy, PEA, Pocket PEA & PEA Plus Software, Hansatech Instruments Ltd.: King's Lynn, England (2006).
18. H. K. Lichtenthaler and J. A. Miehé, "Fluorescence imaging as a diagnostic tool for plant stress," *Trends Plant Sci.* **2**, 316–320 (1997).
19. L. Liu and Z. Cheng, "Detection of vegetation light-use efficiency based on solar-induced chlorophyll fluorescence separated from canopy radiance spectrum," *IEEE J. Sel. Topics Appl. Earth Observ. Remote Sens.* **3**, 306–312 (2010).
20. R. M. Zablotowicz and K. N. Reddy, "Nitrogenase activity, nitrogen content, and yield responses to glyphosate in glyphosate-resistant soybean," *Crop Prot.* **26**, 370–376 (2007).
21. L. H. S. Zobiole et al., "Water use efficiency and photosynthesis of glyphosate-resistant soybean as affected by glyphosate," *Pestic. Biochem. Physiol.* **97**, 182–193 (2010).
22. L. H. S. Zobiole et al., "Glyphosate affects chlorophyll, nodulation and nutrient accumulation of 'second generation' glyphosate-resistant soybean (Glycine max L.)," *Pestic. Biochem. Physiol.* **99**, 53–60 (2011).
23. G. A. Carter et al., "Detection of solar-excited chlorophyll and fluorescence and leaf photosynthetic capacity using a Fraunhofer line radiometer," *Remote Sens. Environ.* **55**, 89–92 (1996).
24. G. A. Carter et al., "Use of a prototype instrument to detect short-term changes in solar-excited leaf fluorescence," *Int. J. Remote Sens.* **25**, 1779–1784 (2004).
25. N. Subhash and C. N. Mohanan, "Curve-fit analysis of chlorophyll fluorescence spectra: application to nutrient stress detection in sunflower," *Remote Sens. Environ.* **60**, 347–356 (1997).
26. F. Zhao et al., "Early detection of crop injury from herbicide glyphosate by leaf biochemical parameter inversion," *Int. J. Appl. Earth Observ. Geoinform.* **31**, 78–85 (2014).
27. H. K. Lichtenthaler and U. Rinderle, "The role of chlorophyll fluorescence in the detection of stress conditions in plants," *CRC Crit. Rev. Anal. Chem.* **19**, 29–85 (1988).
28. K. N. Reddy, R. E. Hoagland, and R. M. Zablotowicz, "Effect of glyphosate on growth, chlorophyll, and nodulation in glyphosate-resistant and susceptible soybean (Glycine max) varieties," *J. New Seeds* **2**, 37–52 (2001).

29. K. N. Reddy et al., "Biological responses to glyphosate drift from aerial application in non-glyphosate-resistant corn," *Pest Manage. Sci.* **66**, 1148–1154 (2010).
30. C. H. Koger et al., "Rice (Oryza sativa) response to drift rates of glyphosate," *Pest Manage. Sci.* **61**, 1161–1167 (2005).
31. F. Zhao et al., "A spectral directional reflectance model of row crops," *Remote Sens. Environ.* **114**, 265–285 (2010).

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