

Diagnosis of Invisible Photosynthetic Injury Caused by a Herbicide (Basta) with Chlorophyll Fluorescence Imaging System

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Abstract

Chlorophyll fluorescence imaging is a powerful tool in obtaining physiological information on plant leaf in non-destructive and non-invasive ways. Effects of Basta, one of the most popular commercially available foliar application-type herbicides, on *in situ* kidney bean leaf was analyzed with a developed chlorophyll fluorescence imaging system. Immediately after the Basta treatment, CO₂ assimilation rate and Φ_{PSII} , which represents the photochemical yield of photosystem II, decreased and NPQ, which represents the heat dissipation of absorbed light energy, increased in the treated area. A positive linear correlation was confirmed between assimilation rate and Φ_{PSII} , while a negative linear correlation was confirmed between assimilation rate and NPQ. These results demonstrated that the early diagnosis of invisible photosynthetic injury caused by Basta was feasible and the chlorophyll fluorescence imaging can be used as a system for early detection of photosynthetic inhibition of growing plants caused by herbicides. [Keywords] chlorophyll fluorescence, CO₂ assimilation rate, heat dissipation, photochemical yield of photosystem II, photosynthetic injury

Introduction

In the controlled greenhouse and precision agriculture, the information on plant response and growth environments measured by sensors is used to achieve the optimization of the controlled system, increase in plant production and sustainable agriculture (Hashimoto *et al.*, 1993, Sigrimis, 2001, De Baerdemaeker *et al.*, 2001). Many studies have been done for the application of biotic information, obtained through non-destructive and non-invasive measuring methods, to diagnose invisible plant responses in the research field. Such an approach is called “Speaking Plant Approach (SPA)” (Hashimoto, 1989). In particular, imaging techniques, e.g. multi- or hyper-spectral image sensing, thermal image sensing, and fluorescence image sensing, have been intensively investigated for use in diagnosis because image information is the most intuitive, easily comprehensible, and provides copious amount of information (Omasa, 1990; Omasa *et al.*, in press). Multi- or hyper-spectral image sensing measures the reflection spectrum from plants, which contains information on pigments and water status in plants (Myers, 1983; Omasa and Aiga, 1987; Omasa, 1990; Omasa, in press). Thermal image sensing measures surface temperature, which provides information on stomatal response and gas exchange (Omasa *et al.*, 1981ab; Hashimoto *et al.*, 1984; Omasa and Aiga, 1987; Omasa, 1990; Omasa and Croxdale, 1992; Jones, 1999; Omasa, 2002). Fluorescence image sensing is an active sensing technique, for example laser-induced fluorescence (LIF) imaging and chlorophyll fluorescence imaging (Omasa 1990; Lichtenthaler, 1996; Omasa, 1998, Govindjee and Nedbal, 2000; Kim *et al.*, 2002; Omasa and Takayama, 2002). These sensing techniques provide

K. Takayama, A. Konishi, and K. Omasa. “Diagnosis of Invisible Photosynthetic Injury Caused by a Herbicide (Basta) with Chlorophyll Fluorescence Imaging System”. *Agricultural Engineering International: the CIGR Journal of Scientific Research and Development*. Manuscript IT 03 001. June, 2003.

information on the bleaching of plant pigments and about photosynthetic functions. Especially, chlorophyll fluorescence imaging, originally developed by Omasa *et al.* (1987) and Daley *et al.* (1989), is a highly sophisticated technique for the evaluation of the heterogeneous distribution of photosynthetic activities, e.g. photosynthetic electron transport, assimilation rate, and stomatal opening over a plant leaf surface (Omasa and Takayama, 2002).

Since chlorophyll fluorescence is emitted from chlorophyll *a* pigments and is the reemission of light energy trapped by antenna chlorophyll not used in photosynthesis, a chlorophyll fluorescence image represents the heterogeneous distribution of light utilization and photosynthetic activity over a leaf surface (Krause and Weis, 1991; Govindjee, 1995). Furthermore, several effective fluorescence parameters indicating the activities of photosynthetic electron transport or heat dissipation of absorbed light energy calculated by "Saturation pulse method" had been developed (Quick and Horton 1984; Genty *et al.*, 1989; Bilger and Björkman, 1990). Taking advantage of the chlorophyll fluorescence imaging, it has been applied to the diagnosis of photosynthetic injury caused by biotic and abiotic stress factors (Balachandran *et al.*, 1994; Mott 1995; Osmond *et al.*, 1998; Omasa and Takayama, 2002). Especially, the diagnosis of invisible photosynthetic injury caused by herbicide with chlorophyll fluorescence imaging has been widely investigated (Genty and Meyer, 1994; Rolfe and Scholes, 1995; Omasa *et al.*, 2001; Takayama *et al.*, 2000; Takayama and Omasa, 2001ab).

In this study, we have demonstrated the early diagnosis of invisible photosynthetic injury caused by Basta with a developed chlorophyll fluorescence imaging system. Basta is the most popular commercially available foliar application-type herbicide, for many genetically engineered crops that have resistance to the herbicide (Lea and Ridley, 1989). Furthermore, the weeding process of Basta on plant leaf was analyzed with continuous measurements of chlorophyll fluorescence imaging.

Materials and Methods

Plant material

Plants of *Phaseolus vulgaris* L. cv. Shin-edogawa were grown in an environmentally controlled growth chamber for 5 weeks after sowing in pots (12 cm in diameter and 10 cm in height). The pots were filled with artificial soil (mixture of vermiculite and perlite, 1:1 v/v). The plants were illuminated for 12 h each day with white fluorescent lights that have a light intensity of approximately PPFD 300 $\mu\text{mol m}^{-2}\text{s}^{-1}$. Air temperature was 26.5 °C during the day and 20.0 °C at night. Relative humidity was 45 % during the day and 60 % at night. Plants were watered daily with a nutrient solution (1:1000 dilution of HYPONex). Mature *in situ Phaseolus vulgaris* L. leaves were used in the experiments.

Herbicide and the treatment

Basta (AgrEbo) is composed of 18.5 % Glufosinate-ammonium <Ammonium -DL-homoalanine-4-YL-(methyl)phosphinate> and 81.5 % mixture of water and detergents. The active ingredient of Basta is glufosinate. It inhibits the activity of glutamine synthetase, which is essential for the removal of toxic ammonia produced in the metabolism of living systems in plants (Lea and Ridley, 1989). The application of glufosinate leads to increased levels of ammonia in the plant tissues. This causes photosynthesis to stop and the plant dies within few days (Lea and Ridley, 1989). In this experiment, a 1:100 dilution of Basta with pure water was used according to label directions for killing annual plants. After the steady-state photosynthesis had been attained under light condition, the measurement of chlorophyll fluorescence and visual

images for the control was done. Thereafter, the herbicide treatment was performed with the following procedure. The upper surface area on the left side of the leaf was covered with a gauze soaked in the Basta dilute solution for 10 min. After the treatment, the measurements of chlorophyll fluorescence and visual images were performed at appropriate intervals for 24 h.

Chlorophyll fluorescence imaging system

Figure 1 shows a schematic diagram of the chlorophyll fluorescence imaging system installed in a laboratory. Two different light intensities, actinic light and saturation light pulse, were used in the chlorophyll fluorescence measurements. The measurement method is called “Saturation pulse method” (Quick and Horton, 1984).

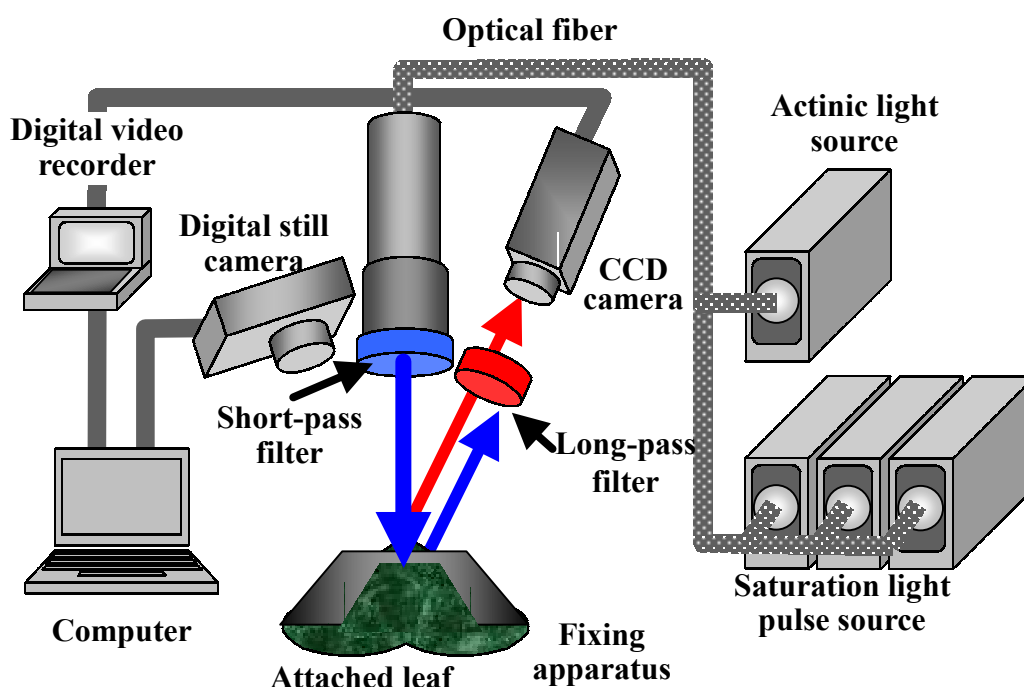


Figure 1. Schematic diagram of chlorophyll fluorescence imaging system. An attached plant leaf was placed on the fixing apparatus. The $5 \times 5 \text{ cm}^2$ measurement area is illuminated by the actinic or saturation light pulse sources. The downward solid blue arrow represents the actinic and the saturation pulse lights transmitted through the short-pass filter. The upward solid blue arrow represents the reflection of the blue illuminations. The upward solid red arrow transmitted through the long-pass filter represents chlorophyll *a* fluorescence emitted from the leaf induced by the blue illuminations.

Actinic light (PPFD $350 \mu\text{mol m}^{-2}\text{s}^{-1}$) for photosynthesis was provided with a 180 W metal halide lamp (Sumita Optical Glass, Inc., LS-M180) equipped with a short-pass filter (Sumita Optical Glass, Inc., Blue-filter; $\lambda < 570 \text{ nm}$) through an optical fiber. Saturation light pulse (PPFD $2700 \mu\text{mol m}^{-2}\text{s}^{-1}$ for 1 s) that caused a transient saturation of photochemistry was provided with three 180 W metal halide lamps (Sumita Optical Glass, Inc., LS-M180) equipped with a short-pass filter (Sumita Optical Glass, Inc., Blue-filter; $\lambda < 570 \text{ nm}$) through the optical fiber. The spectral property of the short-pass filter was represented as the blue solid line shown in Figure 2. The optical

fiber has four small optical fiber branches, one of those branches was connected to the actinic light source and three of those branches were connected to the saturation light pulse sources. The optical fiber collects the blue illuminations provided by the actinic and saturation light pulse sources and provides the blue illumination on an attached plant leaf. The variation in the distribution of the blue illumination within the measurement area ($5 \times 5 \text{ cm}^2$) was kept within less than $\pm 5 \%$.

Chlorophyll fluorescence induced by the blue illumination was captured by a B/W chilled charge-coupled device (CCD) video camera (Hamamatsu Photonics, C5985) equipped with a long-pass filter (Corning, 2-64; $\lambda > 640 \text{ nm}$) in order to remove the reflectance of the blue light illumination. Spectral property of the long-pass filter was represented as the red solid line shown in Figure 2. The chlorophyll fluorescence images were recorded on a digital video recorder (SONY, DSR-V10) as $480\text{H} \times 720\text{V}$ pixels/frame with an 8-bit resolution. Visual changes of plant leaves after the herbicide treatment were observed by visual images obtained with a digital still camera (Sony, DSC-70). The capturing of visual image was performed after a series of chlorophyll fluorescence measurements. After the experiment, the recorded chlorophyll fluorescence images were transferred to a computer and analyzed.

The fixing apparatus for a plant leaf attached to the plant was placed at a distance of 0.12 m from the optical fiber and 0.55 m from the CCD camera. The size of illuminated area was $7 \times 7 \text{ cm}^2$ and the measurement area was $5 \times 5 \text{ cm}^2$ located at the center of the illuminated area. The attached plant leaf was horizontally set on the fixing apparatus normal to the axis of the blue illumination from the optical fiber. Upper and lower surfaces of the plant leaf on the fixing apparatus were opened to the air.

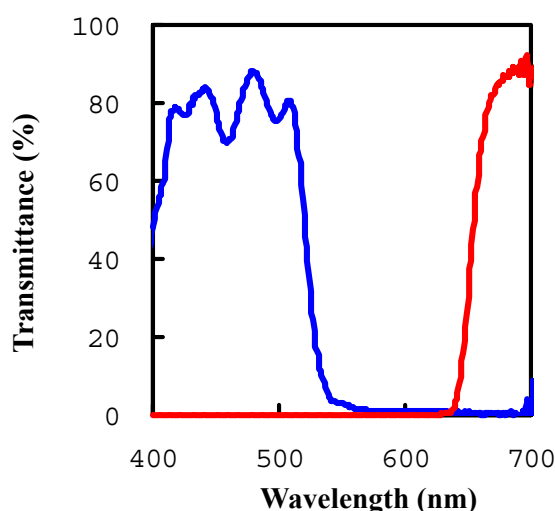


Figure 2. Spectral properties of the optical filters used in the chlorophyll fluorescence imaging system. The blue solid line represents the spectral property of the short-pass filter. The red solid line represents the spectral property of the long-pass filter. Transmittances were measured with MSR-7000.

Chlorophyll fluorescence analysis with “Saturation pulse method”

Chlorophyll fluorescence analysis with saturation light pulse is called “Saturation pulse method” (Quick and Horton, 1984; Genty *et al.*, 1989; Bilger and Björkman, 1990). This method has been applied to the chlorophyll fluorescence imaging analysis (Daley *et al.*, 1989; Genty and Meyer, 1994). Figure 3 shows a schematic diagram of time course of chlorophyll fluorescence imaging and the state of the blue illuminations in the saturation pulse method. First, an attached plant leaf placed in the fixation apparatus was acclimated to a dark condition for at least 1 h. After the acclimation, an F_m image was captured during the saturation light pulse (SL in

Fig.3) by a CCD camera with a shutter speed of 0.03 s. Next, the leaf was illuminated with an actinic light source (AL in Fig.3). After the photosynthesis reached a steady state, an F_m' image was obtained during the saturation light pulse under actinic light by a CCD camera with a shutter speed of 0.03 s. An F image was captured just before the F_m' measurement under actinic light by a CCD camera with a shutter speed of 0.5 s. Nomenclature of fluorescence parameters is according to van Kooten and Snel (1990). The relationship between fluorescence intensity, which was emitted from a fluorescent sheet illuminated with blue actinic light through the optical fiber illustrated in Fig. 1, and the digital output from the camera was calibrated. Figure 4 shows the relationships between fluorescence intensity and analog to digital (A/D) conversion level obtained at the shutter speeds of 0.03 s (● in Fig.4) and 0.5 s (■ in Fig.4) and the regression lines for each shutter speed.

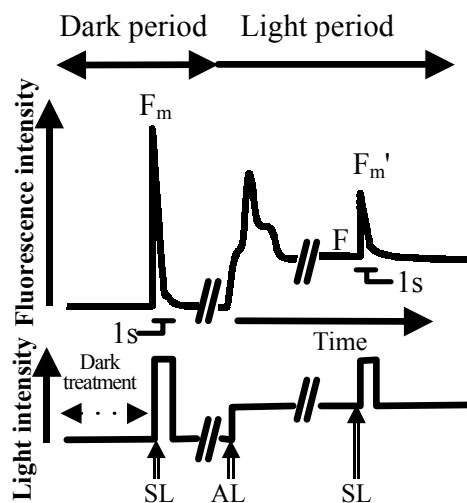


Figure 3. Schematic diagram of time course of “Saturation pulse method”. Dashed arrow represents the dark treatment before the chlorophyll fluorescence imaging. SL represents the irradiation time of the saturation light pulse. AL represents the start time of light period. F_m , F , and F_m' represent the chlorophyll fluorescence imaging time points.

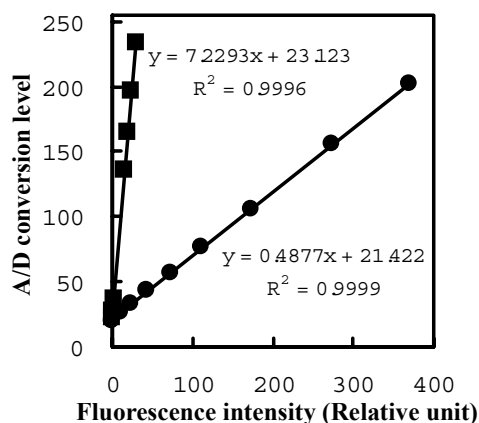


Figure 4. Relationships between fluorescence intensity and analog to digital (A/D) conversion level obtained under two different shutter speed settings, 0.03 s (●) and 0.5 s (■). The regression lines were used in the conversion of the A/D conversion level to the relative fluorescence intensity.

In order to determine photosynthetic properties from chlorophyll fluorescence measurements, the fluorescence intensity images (F_m' , F , and F_m) must be converted into relative fluorescence yield images ($\Phi F_m'$, ΦF , and ΦF_m) with the following correction formulae developed from formulae indicated in Fig.4 and the ratio (Ratio = 7.71) of saturation light pulse intensity to actinic light intensity.

$$\Phi F_m = \frac{F_m - 21.422}{0.4877}$$

$$\Phi F_m' = \frac{F_m' - 21.422}{0.4877}$$

$$\Phi F = \frac{F - 23.123}{7.2293} \times \text{Ratio}$$

Using the relative fluorescence yield images, images of the photochemical yield of photosystem II (PSII) (Φ_{PSII}) and nonphotochemical quenching (NPQ) were computed. Images of Φ_{PSII} obtained under actinic light represent the activity of linear electron transport in PSII and ranged from 0 to 1 (Genty *et al.*, 1989). Images of Φ_{PSII} can be used as relative maps of CO₂ fixation in non-photorespiratory condition and were computed for each corresponding pixel using the following formula (Genty and Meyer, 1994; Rolfe and Scholes, 1995):

$$\Phi_{\text{PSII}} = \frac{\Phi F_m' - \Phi F}{\Phi F_m'}$$

NPQ, ranging from 0 to infinity, represents the extent of intrathylakoid pH gradient and the ability of chloroplasts to dissipate excess excitation energy as heat (Bilger and Björkman, 1990; Osmond *et al.*, 1998). Thereby, NPQ has been used as a sensitive indicator of regulation and protection of photosynthesis in environments when light energy absorption exceeds the capacity for light utilization. Images of NPQ were calculated for each corresponding pixel using the following formula (Osmond *et al.*, 1998):

Images of Φ_{PSII} and NPQ were stored as 480H × 720V pixels/frame with an 8-bit resolution.

$$\text{NPQ} = \frac{\Phi F_m - \Phi F_m'}{\Phi F_m'}$$

Measurement of CO₂ assimilation rate with porometer

In order to evaluate the relationships between CO₂ assimilation rate and chlorophyll fluorescence parameters (Φ_{PSII} and NPQ) of a *Phaseolus vulgaris* L. leaf after the Basta treatment, CO₂ assimilation rate and chlorophyll fluorescence parameters were simultaneously measured with a porometer (PPSYSTEMS, CIRAS-1) and the chlorophyll fluorescence imaging system, respectively. The leaf cuvette equipped with the porometer measured CO₂ assimilation rate within a selected circular leaf area (18 mm diameter) before and after the Basta treatment. The leaf cuvette has a transparent window at the upper side. Hence, chlorophyll fluorescence imaging was concurrently performed within the circular leaf area selected by the cuvette through the transparent window. The measurement was performed at appropriate time intervals in daytime for 2 consecutive days after the Basta treatment. Environmental conditions and experimental protocols were the same as those of chlorophyll fluorescence imaging described above.

Results and Discussion

Figure 5 shows changes in the distribution of chlorophyll fluorescence intensity (F and F_m'), Φ_{PSII} , and NPQ over the leaf surface and visual appearance during 24 h after the Basta treatment (Control, 30 min, 3 h, and 24 h). Image of F_m was captured before the treatment and was used in the calculation for the NPQ image. The treated area was covered with a gauze soaked in 1:100 dilution of Basta with pure water.

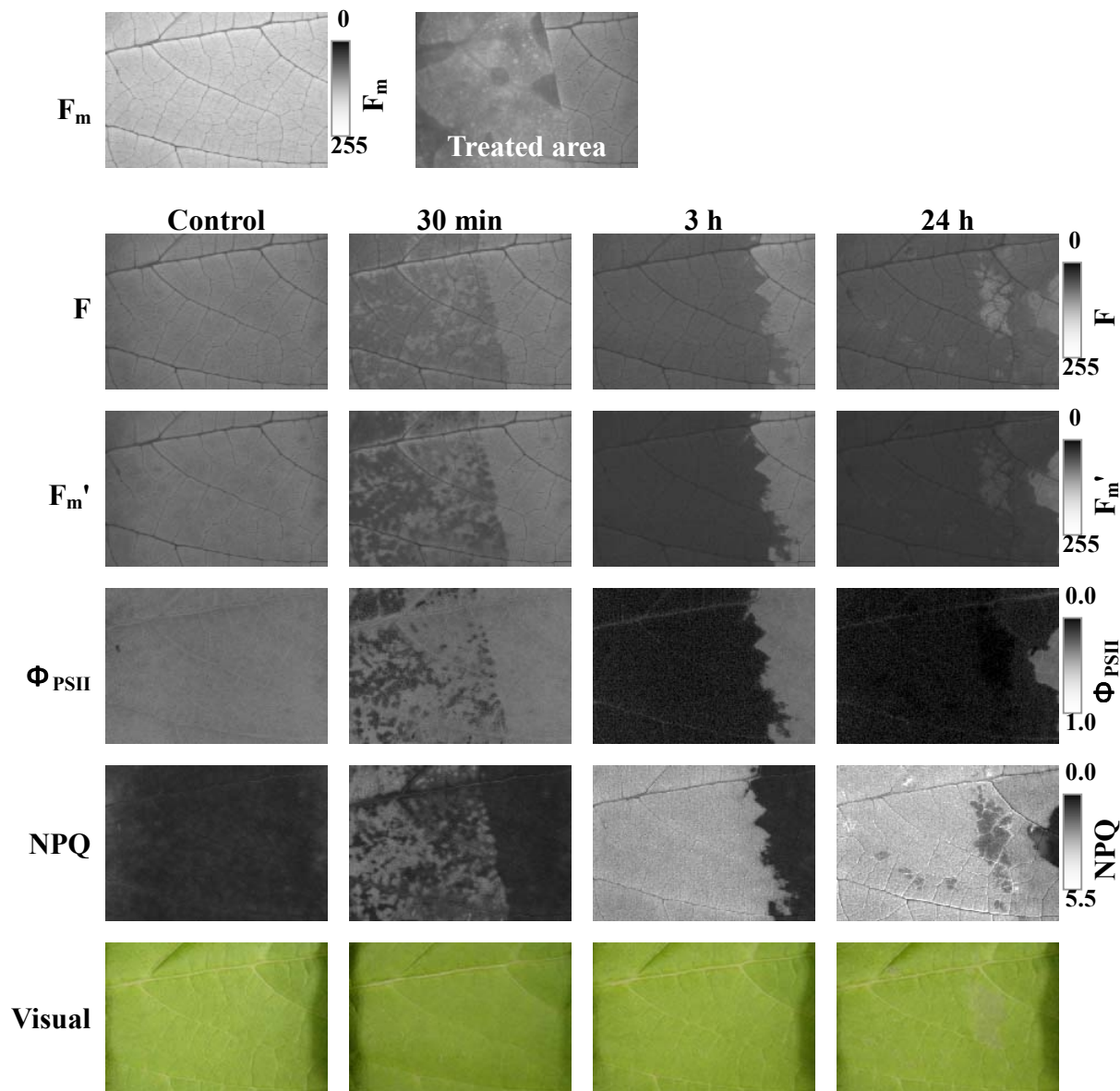


Figure 5. Effects of the Basta treatment on a *Phaseolus vulgaris* L. leaf. The fluorescence image titled “Treated area” represents the Basta treatment. Chlorophyll fluorescence images (F and F_m') were captured before (Control), 30 min, 3 h, and 24 h after the treatment and Φ_{PSII} and NPQ images were calculated at each time.

Before the treatment, chlorophyll fluorescence intensity (F and F_m') was uniformly distributed over the leaf surface except for the major veins and the neighboring areas. Φ_{PSII} and NPQ were also uniformly distributed in those areas and the mean values were

0.44 and 0.96, respectively. The values of Φ_{PSII} and NPQ indicated that the photosynthetic activity in the mesophyll areas was at a moderate level over a leaf surface (Genty and Meyer, 1994; Osmond *et al.*, 1998). At 30 min after the treatment, the patchy decrease in chlorophyll fluorescence intensity (F and F_m') within the treated area was confirmed. In those areas, Φ_{PSII} decreased to 0.25 and NPQ increased to 2.26. The time after the treatment suggested that Basta rapidly moved into mesophyll cells from the leaf surface. Then, it began to inhibit the photosynthetic electron transport activity and activated the heat dissipation pathway of absorbed light energy (Genty and Meyer, 1994; Osmond *et al.*, 1998). The patchiness may be caused by heterogeneous absorption of herbicide during the herbicide treatment through stomata. Uptake of Basta in the areas with low Φ_{PSII} and high NPQ seemed to be larger than in non-injured areas. Thereafter, injured areas were connected with each other and expanded to the adjacent untreated area at 3 h after the treatment. This showed that Basta absorbed in leaf diffused in the leaf apoplast at first and then it was transported along veins and vienlets. The mean value of Φ_{PSII} and NPQ in the injured areas was 0.11 and 3.47, respectively. The values represented that photosynthetic electron transport activity was severely inhibited and the heat dissipation pathway of absorbed light energy was significantly activated in those areas (Krause and Weis, 1991; Genty and Meyer, 1994; Osmond *et al.* 1998). At that time, visual change was not yet observed. At 24 h after the treatment, the injured area further expanded and occupied almost all of the measurement area. The expansion rate between 3 h and 24 h after the treatment, along the major vein was faster than that in mesophyll area. This indicated that the Basta was transferred from the treated area to the untreated area located in the margin side of the leaf predominantly through the major vein and inhibited photosynthetic functions in those areas. The browning areas (visible injury) were recognized in the untreated area located adjacent to the treated area at that time. In those areas, re-increased chlorophyll fluorescence intensity (F and F_m') was confirmed. Then, Φ_{PSII} decreased to 0.01 and NPQ re-decreased to 2.25. This suggested that the pigments emitting the chlorophyll fluorescence still existed, however, both photochemical and nonphotochemical processes were dramatically abolished (Krause and Weis, 1991; Govindjee, 1995). The reason why the visible injury initially occurred in the untreated area and not in the treated area could not be explained in this study. In the affected areas except for the areas with visible injuries, the mean value of Φ_{PSII} and NPQ were 0.07 and 4.03, respectively. In spite of no visible injury in those areas, photosynthetic electron transport activity was severely inhibited, and the heat dissipation pathway was activated (Krause and Weis, 1991; Govindjee, 1995).

Figure 6 shows relationships between CO_2 assimilation rate and Φ_{PSII} (●) and between assimilation rate and NPQ (□) during 24 h after the Basta treatment. When the assimilation rate decreased from $12.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ to $0.3 \mu\text{mol m}^{-2} \text{s}^{-1}$, Φ_{PSII} decreased from 0.34 to 0.01, and NPQ increased from 0.45 to 2.64. A positive linear correlation was confirmed between assimilation rate and Φ_{PSII} , while a negative linear correlation was confirmed between assimilation rate and NPQ. It showed that a low Φ_{PSII} and a high NPQ indicate a low assimilation rate, and a high Φ_{PSII} and a low NPQ indicate a high assimilation rate. These results prove that the assimilation rate in the areas with low Φ_{PSII} and high NPQ in Fig. 5 was low and vice versa.

Figure 7 shows a relationship between Φ_{PSII} and NPQ during 24 h after the Basta treatment. A close inverse correlation was confirmed between Φ_{PSII} and NPQ. This result implies that the decrease in photosynthetic electron transport activity was mainly caused by the activation of heat dissipation of absorbed light energy. These results could be interpreted as follows. First, Glufosinate, the active ingredient of Basta, initially induced the accumulation of ammonia in mesophyll tissues (Lea and Ridley, 1989). Second, it altered the extent of intrathylakoid pH gradient and the

activity of heat dissipation pathway, which resulted in an increase in NPQ (Krause and Weis, 1991; Govindjee, 1995; Osmond *et al.*, 1998). Third, the photosynthetic electron transport activity was reduced and which resulted in a decrease in Φ_{PSII} (Genty *et al.*, 1989; Genty and Meyer, 1994). Under normal growing conditions, Φ_{PSII} does not necessarily relative to CO_2 assimilation because the electrons transported thorough PSII are used for photorespiration and other electron sinks (e.g. Mehler reaction) as well as CO_2 assimilation. In this experiment, however, the decrease in Φ_{PSII} favorably coincided with the decrease in CO_2 assimilation rate after the Basta treatment. This result showed that the activities of photorespiration and other electron sinks were concurrently inhibited by Basta as well as CO_2 assimilation.

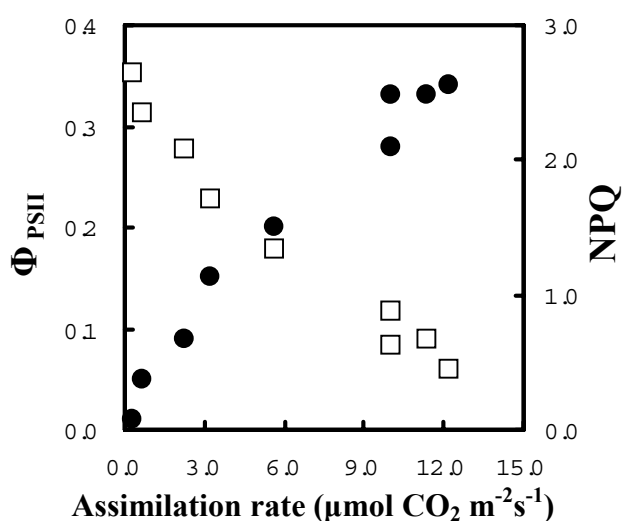


Figure 6. Relationships between CO_2 assimilation rate measured with the porometer measurement, Φ_{PSII} (●) and NPQ (□) calculated from the chlorophyll fluorescence imaging during 2 days after the Basta treatment. The herbicide treatment and environmental conditions were the same as in Fig. 5.

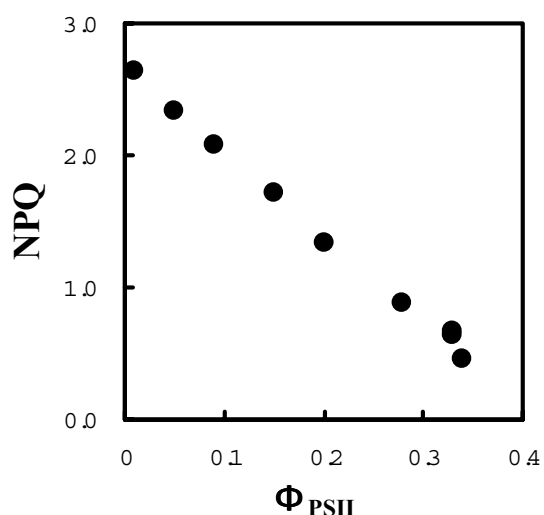


Figure 7. Relationship between Φ_{PSII} and NPQ after the Basta treatment. Φ_{PSII} and NPQ were calculated from the chlorophyll fluorescence images.

Moreover, NPQ also quantitatively evaluated the decrease in CO_2 assimilation rate after the Basta treatment. The NPQ can be calculated only with the saturation light pulse, so this result suggested that the simplified diagnostic procedure using NPQ could be easily applied to the field experiment.

Conclusion

The experiments described here showed that the invisible photosynthetic injuries caused by Basta could be detected at the early stage of the inhibitions by Φ_{PSII} and NPQ images obtained with chlorophyll fluorescence imaging. Furthermore, it also provided topographical diffusion process of Basta in apoplast, veins, and veinlets. The detected injury was identified as a decrease in CO₂ assimilation rate by the porometer measurement performed concurrently with chlorophyll fluorescence imaging.

The Φ_{PSII} and NPQ do not necessarily relative to CO₂ assimilation under normal growing condition. In this experiment, however, the decrease in Φ_{PSII} favorably coincided with the decrease in CO₂ assimilation rate after the Basta treatment. This result showed that the activities of photorespiration and other electron sinks were concurrently inhibited by Basta as well as CO₂ assimilation. Moreover, NPQ also quantitatively evaluated the decrease in CO₂ assimilation rate after the Basta treatment. Consequently, this study provides evidence that the chlorophyll fluorescence imaging proves to be a powerful tool in obtaining biotic information on plant leaf in non-destructive and non-invasive ways.

Acknowledgement

We thank Takateru M. Uenishi and Ryosuke Endo for helpful discussion and comments on the manuscript. We are grateful to Japan Society for the Promotion of Science for funding this study.

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