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Biophoton Emissions in Sulfonylurea-Herbicide-Resistant Weeds

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<http://dx.doi.org/10.5772/55846>

1. Introduction

In the 1950s, a new weed biotype was first reported to be resistant to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) [1]. Since then, the number of weed biotypes resistant to various herbicides has increased dramatically and reported worldwide. There are 393 biotypes of 211 weed species that have evolved resistance to compounds from all the major groups of herbicides [2]. Biotypes of 127 weed species were found to be resistant to herbicides in the acetolactate synthase (ALS) inhibitor group, one of the most successful of the herbicide groups [2].

To control weeds efficiently, it is necessary to identify their herbicide resistance before applying herbicides in the field; however, some herbicides, including ALS inhibitors, act slowly. Therefore, it is difficult to quickly identify whether the weeds in the field are resistant. Recently, some rapid methods for identifying resistance to ALS inhibitors have been developed. For example, an identification method based on an *in vivo* ALS activity assay [3-7] and one based on the regrowth of roots or shoots from weed samples treated with herbicide [8-10] have been developed for identification of sulfonylurea (SU), the major compound group in ALS inhibitors. Here, we propose an alternative method of biophoton measurement for identifying herbicide resistance more simply and rapidly. In this chapter, we introduce the method by providing examples, particularly for detecting SU herbicide-resistant weeds.

2. What are biophotons?

All living organisms spontaneously generate ultraweak photon emissions, commonly referred to as “biophotons,” which originate from biochemical reactions in their cells. Biophotons

can be detected easily, nondestructively, and in real time by using a photon counter equipped with highly sensitive photomultiplier tubes. They have accordingly gained considerable attention as a new tool by which to identify the condition of living organisms. The emission intensity of biophotons is extremely weak at 10^0 – 10^3 photons per second per square centimeter of surface area, and in a nearly continuous spectrum within the optical range of at least 200–800 nm [11, 12]. Biophotons are considered to be associated with the oxidative metabolic reactions essential for life activities, unlike the bioluminescence observed in fireflies, which employs a luciferin–luciferase system. Biophotons were first observed by Coli (1955) in seedlings of plants such as wheat and lentils [13]. Thereafter, this ultraweak biophoton emission was reported by many researchers at the organism, tissue, and cellular levels [11, 14]. It has been suggested that reactive oxygen species (ROS) are the driving force behind biophoton emission because biophotons disappear if the oxygen supply is cut off [15]. The majority of biophotons, if not all, are believed to be emitted in the process of oxidation of substances such as unsaturated fatty acids, amino acids, and polyphenols. These substances are peroxidized and excited by ROS or enzymes such as NADPH oxidase, peroxidase, lipoxygenase, and cytochrome P450. Excited carbonyl compounds generated through this process are considered to be important emitting molecules [15]. In some cases, the excited carbonyl compound is produced as a result of free radical recombination reactions [16]; however, because the quantum efficiency of excited carbonyl is relatively low, the biophotons detected from the living body, to some extent, appear to originate in fluorescent substances in cells, to which the excess energy of excited carbonyl is transferred [17]. In plants, chlorophyll is a good example of this energy transfer. In green seedlings containing large amounts of chlorophyll, biophoton emission was observed only in the range of red light, whereas its original spectrum of biophoton emission was between yellow and orange [18]. In addition, among ROS, only singlet oxygen species emit light. However, except in special cases, the concentration of singlet oxygen in the cell is not sufficiently high to produce biophotons that can be detected with a photon counter [17]. Therefore, the detailed mechanism of biophoton emission in the living body has not been fully clarified. Biophoton research is particularly advanced in the medical field, where photon counters have been experimentally applied to tumor detection [19], brain monitoring [20], and diagnosis of renal failure [21].

3. Biophoton emissions from plants in response to stress

In plants, the most important role of biophotons is to respond to biological (e.g., pathogens, insects, and wounds) and non-biological (e.g., temperature, drought, salt concentration, and chemicals) stimuli to which biophoton increments are primarily observed in plants that acclimatize to these stimuli. Biophoton emission occurs in response to the destruction of cells and as a result of controlled biochemical reactions in cells. Many studies have attempted to detect stress responses in plants using biophotons.

3.1. Response to biological stress

It is well known that pathogenic infections induce a significant increase in biophoton emissions in plants. On the basis of the disease-resistance reactions of plants, biophotons are classified

into two types: the relatively weak emissions observed during the early stages of the resistance reaction [22], and strong emissions from cells exhibiting programmed cell death (PCD) during the middle stages of the resistance reaction to localize the pathogen from healthy cells [23]. In sweet potatoes undergoing PCD as a result of inoculation with *Fusarium oxysporum*, it was reported that the wavelength composition of photon emission considerably shifted toward a shorter wavelength as compared with that of untreated samples, indicating that this was a luminous phenomenon quantitatively different from the one observed under normal conditions [24]. In addition, in the early stage of the resistance reaction, biophoton emission has been proven to occur through the signaling cascade that occurs in resistance reactions [25]. Taking into consideration the first report on the direct link between resistance reaction and biophotons, the subsequent report presents a very important finding. Because of this direct linkage, biophotons were enhanced during the early resistance reaction in plants pretreated with plant activators that enhance the resistance reaction to pathogens [22]. Thus, the two types of biophoton emissions found in disease-resistance reactions are clearly based on controlled biochemical oxidative reactions in cells.

Other than pathogenic infection, exogenously applied plant hormones (gibberellins, auxins, salicylic acid, jasmonic acid) and herbivore damage are reported to induce biophoton emissions [24, 26, 27].

3.2. Response to non-biological stress

Biophoton emission from plants has been reported to dramatically increase under high temperatures that are fatal to plants [28]. This is a good example of biophoton emission in response to cell destruction, in which uncontrolled oxidative reactions occur. In addition, an increase in biophoton emission was observed during acclimation of the plants to high temperature (but not fatal levels) [24]. In contrast, because a transient increase in biophotons is also caused by a sudden decrease in temperature, it has been proposed that this phenomenon can be applied for testing the resistance of plants to frost damage [29].

In addition to the response to temperature, plants also exhibit biophoton emission responding to salt and drought stresses. When azuki bean sprouts were treated with NaCl solution, biophoton emission weakened up to 1 M, but increased at 4.5 M [30]. A mild increase in biophoton emission also occurred under dry conditions, and an extremely strong increase in biophoton intensity was observed when the sprouts that had been dried over many hours were permitted to absorb water again [31]. Thus, under conditions of severe stress that are rarely conducive for the survival of plants, the increment in biophoton emission is a result of the destructive oxidative process of cells.

3.3. Response to herbicide treatment

Chemicals such as herbicides can also act as a non-biological stress for plants. Paraquat is a typical photosystem I inhibitor that acts on the photosynthetic membrane system in photosystem I. Paraquat causes tissue damage by generating ROS through the reduction of molecular oxygen. Intense biophoton emission was observed from the leaves of tobacco plants

treated with paraquat (Figure. 1) [32]. Furthermore, it was confirmed that biophoton emission from leaves treated with paraquat decreased when antioxidants such as catechin were additionally applied [32]. Therefore, it was suggested that the biophoton emission from paraquat treatment was strongly related to the ROS generated in cells.

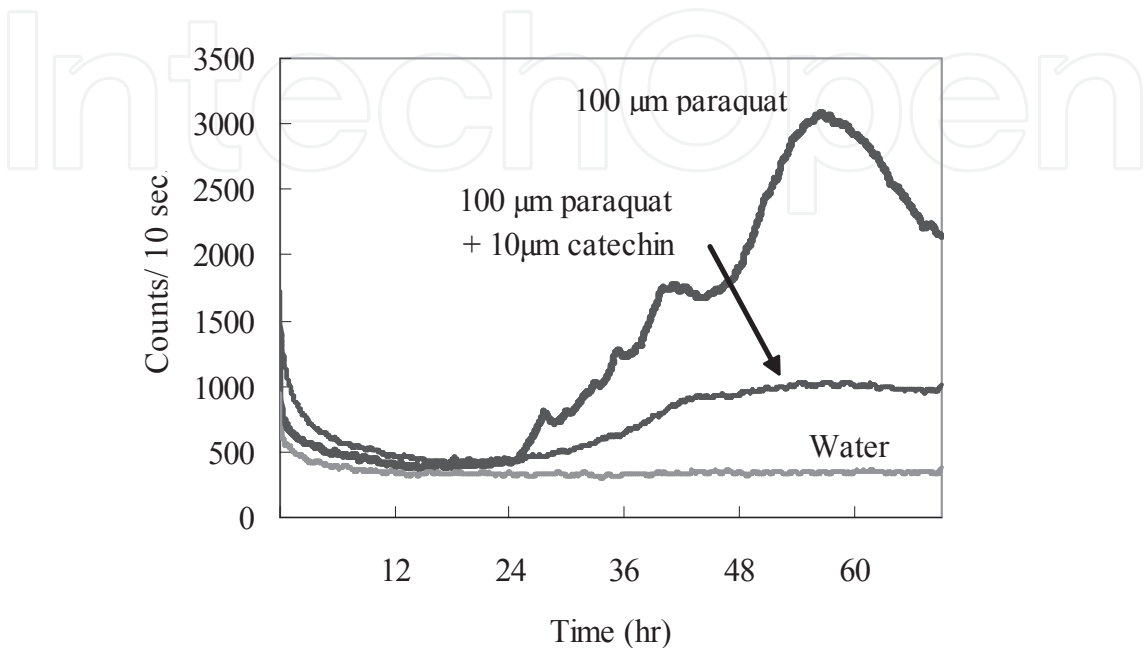


Figure 1. Effectiveness of catechin on biophoton emissions from leaves of tobacco treated with paraquat [32].

The effects of 18 herbicides on biophoton emission from cultured rice cells have been examined (Nukui *et al.*, not published). Among the herbicides investigated, 10 increased the biophoton emissions from rice cells compared with solvent treatment, whereas six decreased emissions, and two had little effect when treated at 100 ppm (Table 1). Some typical results are shown in Figure 2.

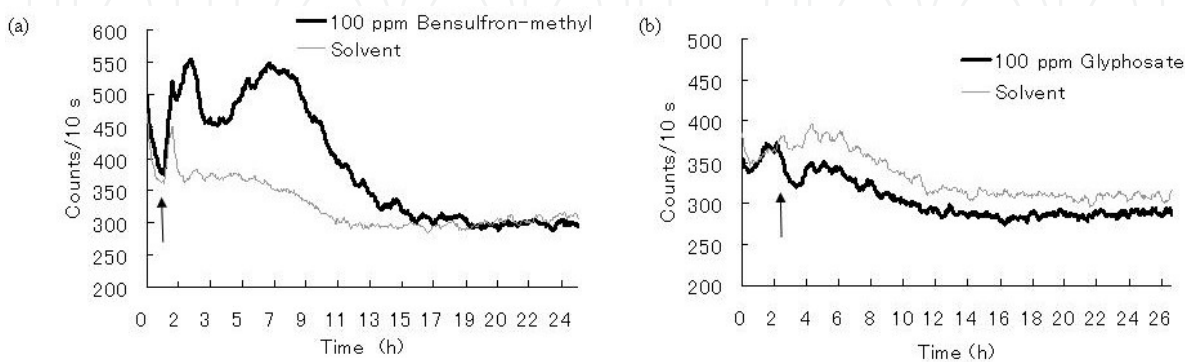


Figure 2. Biophoton emission from cultured rice cells treated with herbicides. (a), bensulfuron-methyl; (b), glyphosate. Values represent the average of duplicates. Arrows indicate the time when cells were treated with the herbicides.

Herbicide	Biophoton ^{z)}	Mode of action
Bensulfuron-methyl	⊙	Inhibition of acetolactate synthase (ALS)
Simetryn	○	Inhibition of photosynthesis at photosystem II
Atrazine	⊙	Inhibition of photosynthesis at photosystem II
Linuron	⊙	Inhibition of photosynthesis at photosystem II
Oxadiazon	○	Inhibition of protoporphyrinogen oxidase(PPO)
Oxadargyl	⊙	Inhibition of protoporphyrinogen oxidase(PPO)
Diflufenican	×	Bleaching: Inhibition of carotenoid biosynthesis at the phytoene desaturase
Amitrole	▲	Bleaching: Inhibition of carotenoid biosynthesis (unknown target)
Glyphosate	▲	Inhibition of EPSP synthase
Glufosinate-ammonium	△	Inhibition of glutamine synthetase
Asulam	×	Inhibition of DHP (dihydropteroate) synthase
Dithiopyr	△	Microtubule assembly inhibition
Trifluralin	△	Microtubule assembly inhibition
Propham	⊙	Inhibition of mitosis/microtubule polymerization inhibitor
Isoxaben	⊙	Inhibition of cell wall (cellulose) synthesis
Dinoterb	⊙	Uncoupling (Membrane disruption)
Dichlorprop	⊙	Synthetic auxins (action like indoleacetic acid)
Pyributicarb	△	Unknown

z) ⊙, increased remarkably; ○, increased; △, no effect; ▲, decreased; ×, decreased remarkably

Table 1. Effects of herbicides on biophoton emissions from cultured rice cells

Herbicides such as bensulfuron-methyl (BSM) induced an increase in biophoton emission from rice cells and then a decrease to control levels within 12–24 h after herbicide application (Figure 2). Although the precise mechanisms of biophoton emission in these cases (except for BSM treatment) were not investigated, these changes must reflect the biochemical reactions of rice cells to these chemicals. As described in detail below, in BSM-treated plants, the oxidative detoxification of BSM by P450 is responsible for biophoton emission.

4. How to measure biophotons from plant segments

4.1. Apparatus for biophoton measurement

Biophotons can be detected using a photon counter equipped with a highly sensitive photo-multiplier tube. In our laboratory, we mainly use multi-sample photon counters, namely,

PCX-100 and CCSPC-01 (Hamamatsu Photonics, Hamamatsu, Japan) (Figure 3). The PCX-100 counter was equipped with a photomultiplier tube (R329; Hamamatsu Photonics, Hamamatsu, Japan) that provided a spectral response from 240 to 630 nm. It has a sample holder for 16 Petri dishes in the dark box and as the photomultiplier moved onto the samples, the biophotons from the samples were measured in rotation. The CCPPS-01 counter was equipped with a photomultiplier tube (R331P; Hamamatsu Photonics, Hamamatsu, Japan) that provided a spectral response from 300 to 650 nm. In the CCSPC-01 system, the photomultiplier was fixed, and a disc with 24 samples rotated under the photomultiplier. Because temperature affects biophoton emissions through e.g. changes in enzymatic activities, the photon counter was placed in an air-conditioned room.



Figure 3. Appearance of photon counter CCSPC-01

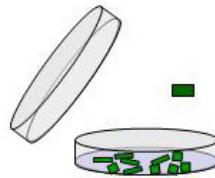
4.2. Sample preparation

Samples such as cut plant segments and cultured cells were used with the PCX-100 and CCSPC-01 counters for biophoton measurements. The procedure using cut plant segments treated with herbicides with the CCSPC-01 counter is shown in Figure 4. Plants were cut into 5-mm-long segments, and 0.5 g of these segments were set in the Petri dishes (60 mm in diameter), to which 2 mL of appropriate concentrations of herbicide solution or solvent (e.g., distilled water) were added. Dishes were then set in a sample holder in the dark box of the photon counter. Biophotons from each sample were continuously measured every 10 s. Because biophotons are luminescent from biological reactions, no special reagent was needed for their detection.

1. Collect and cut the target plants into 5-mm-long segments.



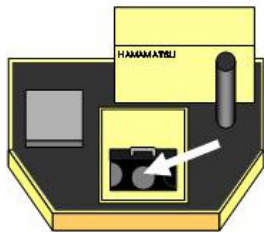
2. Set 0.5 g of the segments in the Petri dishes (60 mm in diameter).



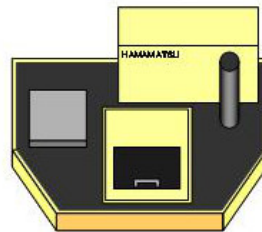
3. Add 2 mL of herbicide solutions or solvent.



4. Set the dishes into sample holder in the dark box of the photon counter.



5. Shut the lid of the dark box and start measuring.



6. Wait about 40 h and analyze the data.

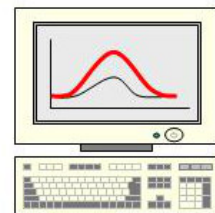


Figure 4. The procedure of measuring biophotons with the CCSPC-01 counter using plant segments treated with herbicide.

It is preferred that all steps after sample collection are performed in a dark room because fluorescence from plant segments, or the Petri dishes due to excitation with room light during the sample preparation, contribute to measurement “noise”.

5. Biophoton emissions in sulfonylurea herbicide-resistant weeds

5.1. Sulfonylurea herbicide resistance

Weed biotypes resistant to ALS-inhibiting herbicides have been reported worldwide [33], and have increased to 127 species [2]. Sulfonylurea (SU) herbicides are among the most potent ALS-inhibiting herbicides used worldwide, and biotypes resistant to SU herbicides have been found in many weed species. For example, *Scirpus juncooides* Roxb. var. *ohwianus* T. Koyama has evolved resistance to herbicides used in the paddy fields of Japan [34, 35], and the resistant biotypes cause serious problems for weed control in Japanese rice production [36, 37]. Mutations in the ALS genes, the target site of SU herbicides, have been reported as the molecular basis of SU resistance in weeds [36]. *S. juncooides* is reported to have at least two ALS genes, and an amino acid substitution at Pro197 or Trp574 in either of the two ALS proteins encoded by the two genes in all the examined resistant biotypes. This type of resistance is referred to as “target-site resistance.”

Another type of herbicide resistance is referred to as “non-target-site resistance.” Mutations in a non-target site, such as activation of herbicide metabolism or reduction of herbicide

absorption and translocation are the molecular bases underlying this type of herbicide resistance. For example, it has been reported that there is SU tolerance in cut leaves in rice and barnyardgrass (i.e., the metabolic half-time of SU was 2.6–4.8 h in rice and 12–50 h in barnyardgrass compared to 50 h or more in susceptible weeds) [38]. Herbicide-resistant weeds with non-target-site resistance have become a more serious problem than those with target-site resistance because non-target-site resistance tends to cause multiple herbicide resistance [39, 40].

5.2. Biophoton emissions in SU-resistant plants through target-site resistance

Biophoton emissions in *S. juncooides* have been investigated by employing 12 biotypes (seven SU-resistant and five SU-susceptible plants) collected from paddy fields in various regions of Japan [41]. Typical results of biophoton emissions from the culms of *S. juncooides* treated with BSM, one of the SU herbicides most commonly used in paddy fields in Japan, is shown in Figure 5 [41].

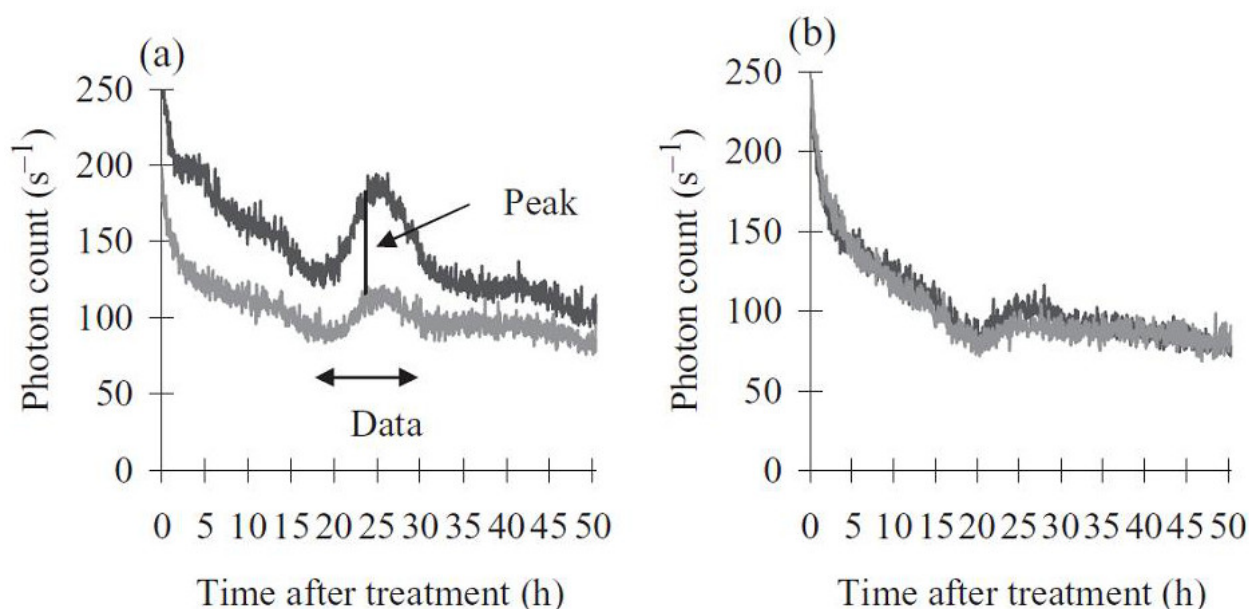


Figure 5. Examples of a typical time course of biophoton emissions from the culms of *Scirpus juncooides* treated with bensulfuron-methyl (BSM) and water. (a) Typical resistant biotype and (b) typical susceptible biotype. The black line indicates the 100 ppm BSM treatment and the gray line indicates the water control [41].

It was observed that the resistant biotypes exhibited an increase in biophoton emissions with a peak at 25 h after 100 ppm BSM treatment. The emission intensity of the biophoton emissions varied depending on the concentrations of BSM. The difference in biophoton emission between resistant and susceptible biotypes is obvious with 10 and 100 ppm BSM treatment, whereas there were few differences with 1 ppm BSM treatment [41]. The emission intensity was different between plant organs (Figure 6) [41]. The increment in the biophoton emission was greater for the culms than for the roots. In the resistant biotype, a relatively higher increment in biophoton emission was observed in both the culms and roots, while the increment was

lower in the susceptible biotypes. This increment of biophoton emission in a resistant biotype was independent of the seed source or mutations in the ALS genes [41]. All seven resistant biotypes showed a higher increment in biophoton emissions than the five susceptible biotypes. The biophoton emissions in the resistant biotype were higher than those in the susceptible biotypes from the vegetative growth to the flowering stage. In particular, the difference in biophoton emission between the resistant and susceptible biotypes widened during the vegetative growth stage, and reached the maximum level at the flowering stage. However, at the seed-setting stage, the biophoton emissions were markedly enhanced in the susceptible biotypes. Therefore, at this stage, there were no distinct differences in biophoton emissions between the resistant and susceptible biotypes [41].

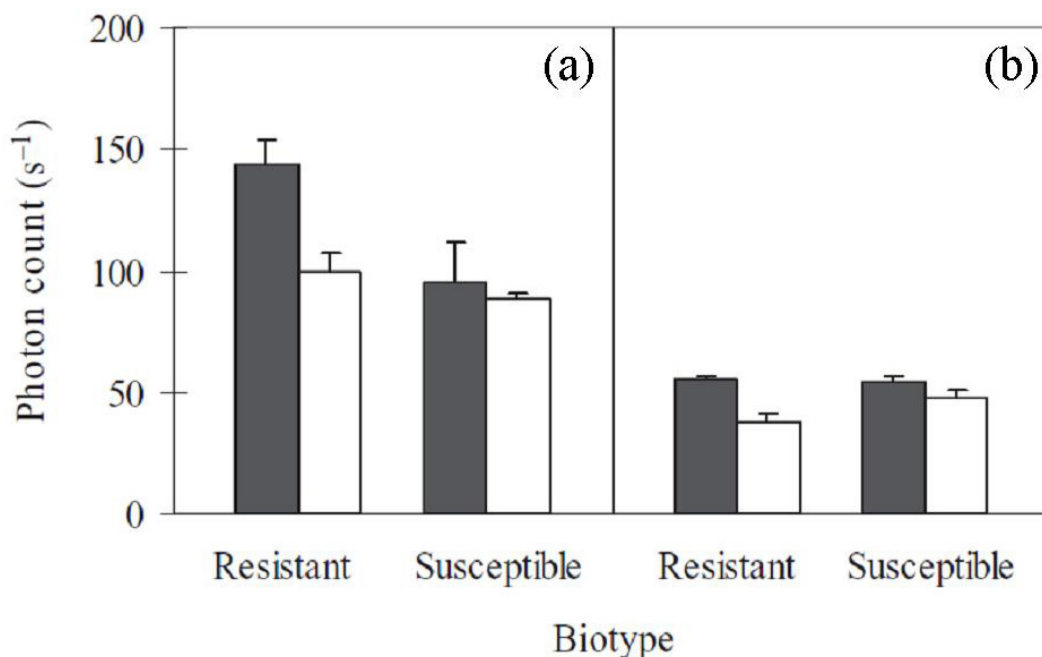


Figure 6. Biophoton generation in the (a) culm and (b) roots of resistant and susceptible biotypes of *Scirpus juncoides*. The bars indicate standard deviations (\pm SD). Bensulfuron-methyl treatment (solid column); control (open) [41].

In addition to the case of *S. juncoides*, this increment in biophoton emission in SU-resistant biotypes has been confirmed in *Monochoria vaginalis* [42]. Figure 7 shows the result of biophoton emissions after BSM treatment from leaf segments of four SU-resistant and four SU-susceptible biotypes of *M. vaginalis* [42]. In the four resistant biotypes, distinct increments were independent of differences in the mutation sites of the ALS genes. In contrast, increments in the four SU-susceptible biotypes were less than that in the four SU-resistant biotypes. Therefore, it is suggested that biophoton emission could be a new indicator of SU-resistant biotypes in various weeds.

5.3. Biophoton emission in SU-resistant plants through non-target site resistance

For biophoton emission in SU-resistant plants through non-target site resistance, biophoton emissions from leaf segments of rice and 11 paddy weeds treated with BSM were measured [43]. There was a definite difference in biophoton emission among plant species, and rice and

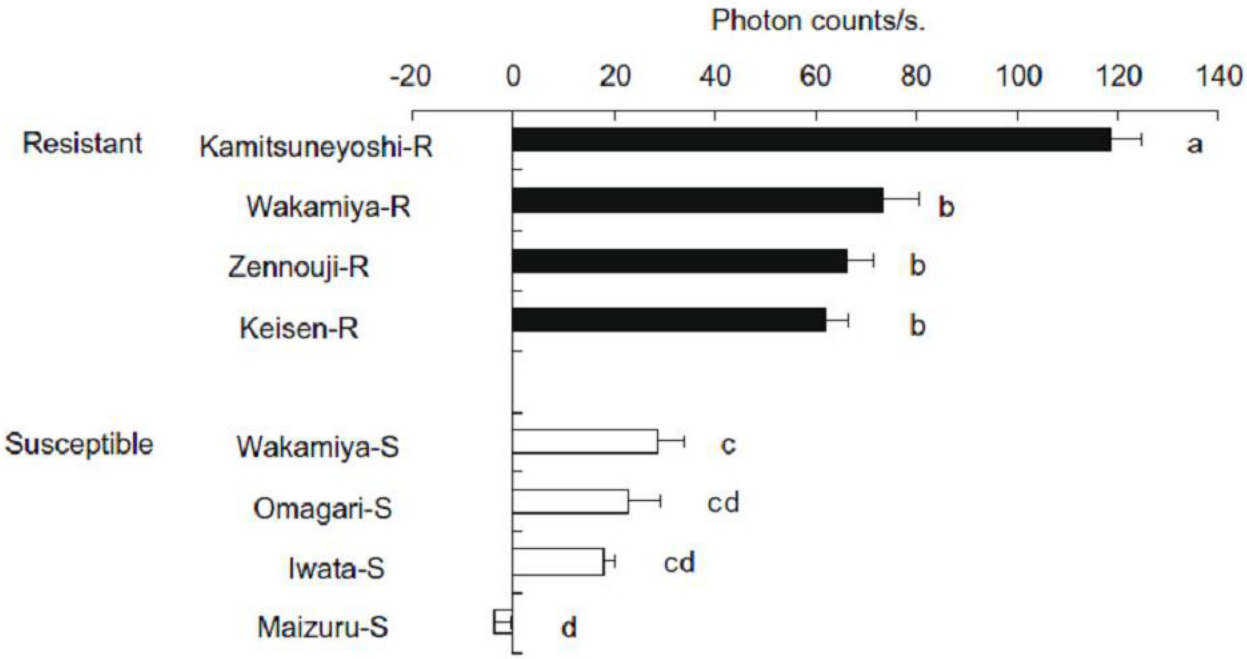


Figure 7. Biophoton generation from leaf segments of resistant and susceptible biotypes of *Monochoria vaginalis*. Values are the differences in averages from 24 to 40 h after treatment between the bensulfuron-methyl application and water control. The bars indicate standard deviations (\pm SD). Different letters indicate a significant difference at the 5% level according to Tukey’s Studentized Range Test [42].

barnyardgrass, which are reported to be tolerant of SU herbicide as described above, showed considerably higher biophoton emission than those of other weed species. The enhancement of biophoton emission in BSM-treated leaf segments compared to the water-treated control was 3.8 times higher in rice and 3.0 times higher in barnyardgrass [33]. It seems that the distinct differences in biophoton emissions among rice, barnyardgrass, and other weed species are consistent with their selectivity against SU herbicides.

6. Mechanism for biophoton emission in plants treated with SU herbicides

Chemical reactions such as oxidation are considered to be the source of energy for biophoton emissions. We previously studied biophoton emissions during plant disease response, particularly with regard to the involvement of ROS. During the disease response of cultured rice cells to *N*-acetylchitoooligosaccharide elicitor, ROS seemed to be directly involved in biophoton emission [25, 44].

As shown in Figure 1, ROS is also involved in biophoton emission in paraquat-treated tobacco. Hideg and Inaba (1991) demonstrated that the leaves of paraquat-resistant tobacco treated with paraquat emitted weaker biophotons compared with tobacco plants sensitive to paraquat [45]. The superoxide dismutase (SOD) activity of the resistant plant was 3 to 6 times greater than that of sensitive plants. Apparently, higher SOD activity in

resistant tobacco contributes to the elimination of the ROS induced by paraquat, resulting in weaker biophoton emission.

Recent studies suggested that oxidative metabolism by cytochrome P450 monooxygenase (P450) might be involved in biophoton emissions from herbicide-treated plants [42, 43]. P450s are a class of heme-containing enzymes that catalyze the biosynthesis of lignin, terpenoid, alkaloids, and many secondary compounds in plants. P450s are also known to play important roles in metabolizing herbicides, including SU [46,47], and are involved in non-target site herbicide resistance [48].

As described above, leaf segments of non-target site resistant plants such as rice and barnyard-grass, emit strong biophotons when treated with SU herbicide, and this biophoton emission was inhibited by the P450 inhibitor (malathion and piperonyl butoxide) treatment [42].

The involvement of P450s in biophoton emission from SU herbicide-treated plants was also confirmed in another experiment employing P450 gene-silenced cultured rice cells. P450s are known to make up one of the largest superfamilies of enzymes and catalyze diverse reactions in both animals and plants. In rice, *CYP81A6*, a P450 gene responsible for resistance to BSM, has been identified by map-based cloning [49]. We silenced the *CYP81A6* gene in rice cells and analyzed their response to BSM (Nukui *et al.*, unpublished). P450-silenced cells showed increased BSM sensitivity as expected. As shown in Figure 2, cultured rice cells, as well as leaf segments, emit biophotons when treated with BSM. Treatment with 100 ppm BSM induced an obvious biphasic biophoton emission in the control cell line. In contrast, P450-silenced cells did not show biophoton emission after BSM treatment. The enhancement of biophoton emission in BSM-treated cells compared to the water-treated control was 1.6 times greater in the control line and 1.0 times in the P450-silenced cell line (Figure 8). Moreover, the effects ALS inhibition on BSM-responsive biophoton emissions have been investigated. BSM inhibits the reaction of ALS, the first step in the biosynthesis of branched-chain amino acids (BCAA: Valine, Leucine, Isoleucine) [50], and the herbicidal activities of BSM is canceled by the application of BCAA [51]. Although the externally applied 1 mM BCAA recovered the cell growth of BSM-treated P450-silenced rice cells, it failed to recover BSM-responsive biophoton emissions. These results suggest that it was not decreased cell activity but the suppressed detoxifying reaction of P450 that reduced biophoton emissions in P450-silenced cells.

On the other hand, the fate of SU compounds is not well known for the target site-resistant weeds such as *S. juncooides* and *M. vaginalis* described above. There are only suggestive results. Figure 9 shows the effect of the P450 inhibitor malathion on biophoton emissions from leaf segments of *M. vaginalis*, which have been confirmed to be target site resistant to SU [42]. In resistant biotypes, malathion decreased biophoton emission from leaf segments treated with BSM, whereas in susceptible biotypes, malathion had no definite inhibitory effect on biophoton emission. This indicates that P450s are also involved in biophoton emissions from target site SU-resistant weeds. Therefore, we hypothesize that SU, which cannot bind to ALS because of ALS mutations, would also be detoxified with P450s, resulting in biophoton emission.

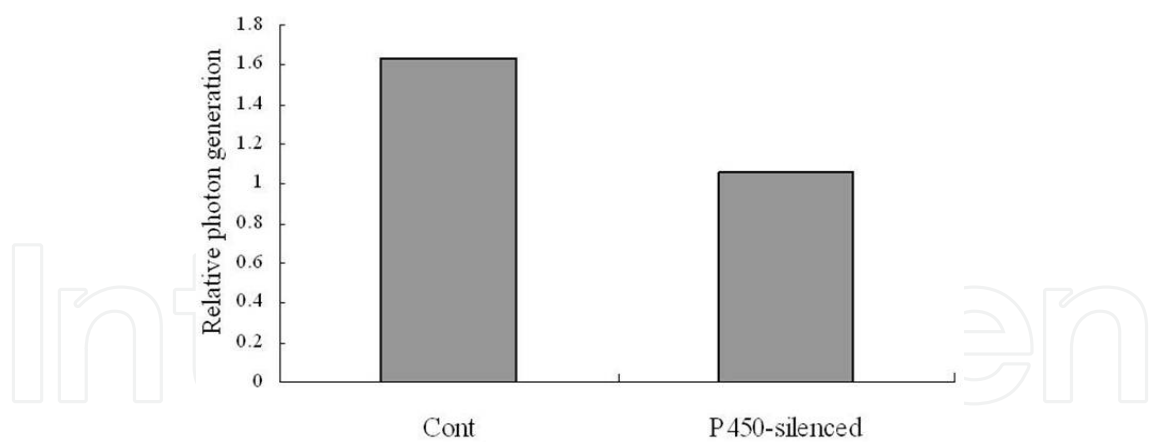


Figure 8. Biophoton emission from P450-silenced rice cells (P450-silenced) and control cells (Cont). Values represent the ratio of total biophoton emission from BSM-treated cells to that from solvent (control)-treated cells during 0-12 h after BSM or solvent application.

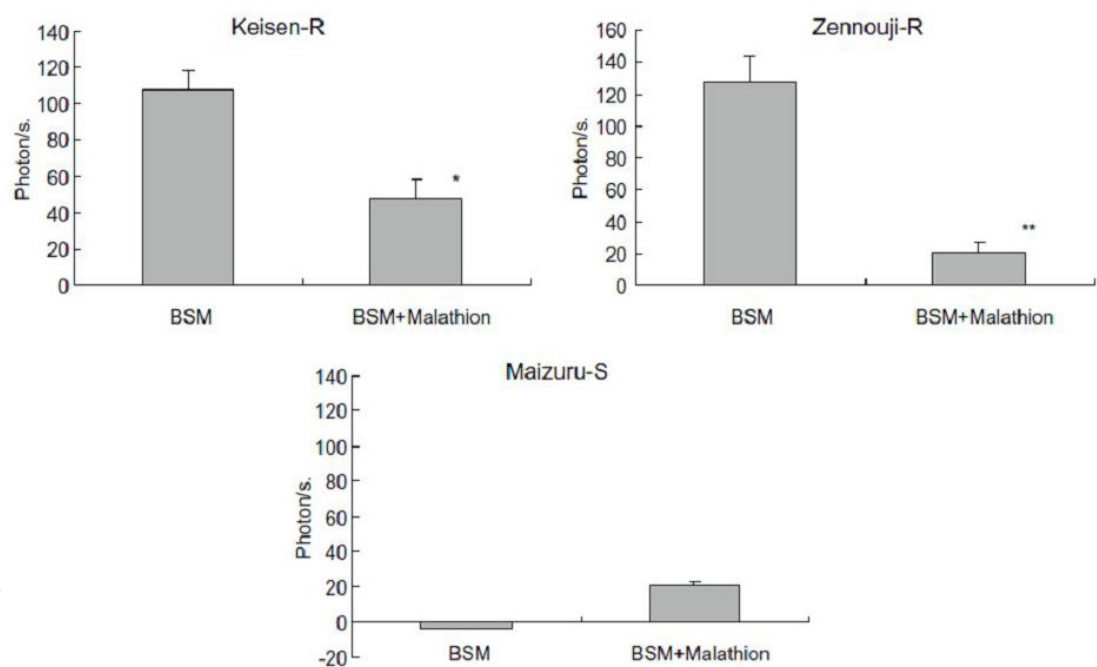


Figure 9. Effect of P450 inhibitor on biophoton emissions from leaf segments of *Monochoria vaginalis*. Values are the differences in averages 24–40 h after treatment between the bensulfon-methyl (BSM) or BSM + P450 inhibitor (malathion) and water control, respectively. The bars indicate standard deviations (\pm SD). * and ** indicate the significant differences at $P < 0.05$ and 0.01 , respectively [42].

7. Conclusion

As described above for SU resistance, it is confirmed that both target site-resistant and non-target site-resistant plants can be distinguished from susceptible plants. In addition, from the analysis of the mechanisms of biophoton emissions, it is considered that weed biotypes with target site resistance to herbicides other than SU might also be distinguished from susceptible

biotypes by biophoton measurement, as long as the herbicides are subjected to detoxification by P450 enzymes. It is well known that P450s play major roles in the detoxification of many types of herbicides, and many resistant weed biotypes with enhanced P450 activities have been reported [48]. For example, rigid ryegrass has been reported to be resistant to diclofop-methyl, diuron, atrazine, simazine, and chlorsulfuron for resistance mediated by P450s [48]. Biophoton measurement might be able to distinguish all these resistant biotypes from susceptible biotypes.

Although it is necessary to clarify the applicable range of herbicide resistance by using weed biotypes resistant to herbicides other than SU, our study suggests that biophoton measurement can be used in the identification of a broad spectrum of herbicide resistances and will become a useful tool for efficient weed control.

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