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A New Method to Evaluate the Stress Intensity of Macrophytes

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Abstract
The physiological condition of aquatic plants, growth and survival rates, and colony formation are all affected by abiotic stresses in nature. However, in ecohydraulics, other factors than dynamic process often do not be considered in a flume experiment or field observation, as there is not any suitable method to identify other stressors effect quickly, except of a long term monitoring of growth rate of plants. Subject to environmental stresses, hydrogen peroxide (H_2O_2), a major reactive oxygen, is generated in the plant cell, associated with the stress intensity, and damages the plant. In the laboratory and field observation, several submerged species were sampled, then H_2O_2 contents were measured together with the major stress intensities, such as temperature, flow velocity, and light intensity, both in light-exposed and dark-adapted conditions. Then, the leaf H_2O_2 concentration of the plant was analyzed. The amount of H_2O_2 increases with increasing each stress intensity, and when multiple stresses are loaded the total stress is given by the sum of contributions of each stress. When the H_2O_2 concentration exceeds a threshold value, the plant deteriorates and finally dies out in several days. With this indicator, the effect of different types of stresses can be compared among others, and the tolerance of the plant is evaluated in a short period of time. This process is available to understand the unpredictable effects in ecohydraulics experiments.

Keywords: Abiotic stress monitoring; Hydrogen peroxide; Oxidative stress; Vegetation management; Ecohydraulics

1. INTRODUCTION

In ecohydraulics, various types of studies are conducted to obtain the macrophytes behaviors with flume experiments and field observations (Carus et al. 2022; Tahorn, et al. 2022; Lama et al. 2022; Villanueva, et al. 2022; Paul and Kerpen 2022; Vetori and Rice, 2020). In most cases, the target is focused on the dynamic processes only, instead of an excellent experimental setup with high cost. Traits related to physiological condition is desirable to obtain even by flume experiments. However, real plants must be used rather than artificial plants in such cases. In the current system, only long-term monitoring of plant traits, such as growth rate and biomass, is commonly employed to obtain whether the plants can grow or die, or whether their colonies can be established (Barko et al., 1991; Riis et al., 2012; O'Hare et al., 2018). One of the reasons is that there is no other method to evaluate the plant's physiological/ecological condition in a short period of experiments. There are various types of effective stresses affecting the plant physiology/ecology in nature. In flume experiments, we need to keep a steady condition of other factors for a suitably long period, even if we look at the effect of drag force, only. In fields, environmental conditions frequently change and it is difficult to keep the same condition.

When plants are subjected to adverse environmental conditions, reactive oxygen species (ROS) are generated in different organelles (Das and Roychoudhury, 2014; Choudhury et al., 2017; Parveen et al., 2017; Asaeda et al., 2018) relatively quickly, which damages the plant body by the oxidative stress (Figure 1). Some ROS are scavenged relatively quickly by antioxidants (Omar et al., 2012), and the homogeneity of ROS in tissues is maintained by balancing the ROS and antioxidants. However, the balance flips over when oxidative stress surpasses the scavenging capacity of the antioxidants (Dumont and Rivoal, 2019). Among ROS, Hydrogen peroxide (H_2O_2) is widely generated (Asada, 2006; Sharma et al., 2012). H_2O_2 is relatively stable and can be quantified easily, compared with other ROS (Satterfield and Bonnell, 1955; Zhou et al., 2006; Asaeda et al., 2020). The concentration of H_2O_2 in plant tissues depends on the stresses associated with their magnitudes if the plant is under unpreferable environmental conditions (Suzuki et al., 2014; Asaeda et al., 2020). Thus, H_2O_2 concentration in the plant tissue can be used as an instantaneous indicator of the physiological status of a particular macrophyte (Smirnoff and Arnaud, 2019; Asaeda et al. 2020). The system has been used for *Egeria densa*, which has successfully identified the channel slope that it can colonize (Asaeda et al., 2020, 2021), riparian terrestrial plant species (Asaeda, et al 2022b), and cyanobacteria under photoinhibition and phosphorus deficiency (Asaeda et al 2022c).

The main objective of the present study is to 1) establish a methodology to determine the H_2O_2 concentration generated by unpreferable conditions of abiotic environmental factors, and 2) to develop an indicator to understand the feasibility of flume experiments and field observation conducted in ecohydraulics.

2. METHODOLOGY

In the field experiment, major submerged species in Japanese rivers, (*Egeria densa*, *Elodea nuttallii*, *Ceratophyllum demersum*, *Potamogeton crispus*, and *Myriophyllum spicatum*) were tested (MLIT, 2019). They were exposed to different types of physical conditions, temperature, irradiance, and water flow velocity, following the range of the rivers where these species were colonized from 5° C in winter to 30° C in summer for water temperature, 0–1,200 µmol/m²/s for the irradiance in water, and 0–50 cm/s for flow velocity (MLIT, 2019). For the laboratory experiments, healthy macrophyte stocks were collected from the Saba River in western part of Japan (*E. densa*) and the Moto-Arakawa River near Tokyo (*E. nuttallii*, *C. demersum*, *P. crispus*, and *M. spicatum*). Collected plants were cleaned with water to remove debris, and any attached macro-algae were carefully separated with tweezers. The plants were then cultured in a glass tank at 25 ± 2° C under a 12/12 h photoperiod with photosynthetically active radiation (PAR) (~125 µmol/m_2/s) using fluorescent lamps) for over 2 months. Commercial sand (D_{50} < 0.1 mm) was used as a substrate, and 5% Hoagland solution was provided as the nutrient medium (Atapaththu and Asaeda, 2015). Algae were removed weekly, and algae-free plants were used in the experiments. Three types of experiments (triplicate) were conducted in small aquaria (dimensions: 50.0 cm × 35.0 cm × 35.0 cm) or in an outdoor flume, where pre-aerated tap water was circulated by centrifugal electric motor pumps. Each experiment focusing on different combinations of environmental factors.

The increment of H_2O_2 concentration of the plant tissue was identified under different water temperatures at (10 ± 2°C (*E. densa*), 15 ± 2°C (*E. densa*), 20 ± 2°C, 25 ± 2°C (*E. densa*), and 30 ± 2, 35 ± 2°C) and irradiance levels (0–1,300 µmol/m_2/s of PAR, to make empirical relations between these factors. The length of the plants grown in the experimental units was measured using a millimeter scale at 5–7 day intervals. The relative shoot growth rate (RGR) was calculated as the difference in shoot length between two observations divided by the duration.

Several rivers that are highly colonized by submerged macrophytes were selected from the species distribution database in Japan (MLIT, 2019). Sampling was conducted in the Eno River and its tributaries (April, May, and September 2016; April and June 2017); in the Saba River and its tributary Shimaji River (May, June, and September 2016; April and June 2017; August 2018), and in the Hii River (October 2016), the Moto-Arakawa River near Tokyo (April 2015). In August 2017, a sampling of *M. spicatum* was conducted in the Sakuradabori of the Imperial Palace Moat, at the center of Tokyo, where *M. spicatum* made a monospecific stand (Asaeda et al, 2020, 2021).

At each sampling point, flow velocity was measured with an ultrasonic velocimeter (Tokyo Keisoku Co. Ltd., Japan) at 20% (reference velocity) and 80% (depth of the colony) of the total water depth (Chow, 2009). The intensity of photosynthetic active radiation (PAR) in the water was measured with a portable quantum flux meter (Apogee, MQ-200, United States) at 10 cm depth intervals. Sampling was conducted in light-exposed and dark-adapted conditions to remove the effect of solar radiation. The dark treatment was created by placing a black plastic sheet (3 m × 3 m) floating over part of the plant colony for 30 min. Then the difference between the light-exposed and dark-adapted samples was considered the effect of light exposure. The 30 min pre-dark period was determined by laboratory experiments, which were specifically conducted to determine the optimum pre-darkness duration (data not shown). The plant samples were placed in plastic bags and immediately stored in a cooling box containing dry ice for transfer to the laboratory where it was stored at −80° C until an H_2O_2 assay and chlorophyll estimation were conducted.

Sampled fresh plant shoots were extracted (~50 mg) in an ice-cold phosphate buffer (50 mM, pH 6.0) that contained polyvinylpyrrolidone (PVP), and the extractions were centrifuged at 5,000 × g for 20 min at 4° C. This extraction was used to analyze the H_2O_2 content spectrophotometrically following the $Ti(SO_4)_2$ method (Satterfield and Bonnell, 1955) with modifications. The reaction mixture contained 750 µL of enzyme extract and 2.5 ml of 1% $Ti(SO_4)_2$ in 20% H_2SO_4 (v/v), which was centrifuged at 5,000 × g for 15 min at 20°C. The optical absorption of the developed yellow color was measured spectrophotometrically at a wavelength of 410 nm. The H_2O_2 concentration in samples was determined using the prepared standard curve for known concentration series and was expressed in µmol per gram fresh weight (µmol/gFW). Chlorophyll a (Chl-a) concentrations of experimental plants were determined spectrophotometrically (UV Mini 1210, Shimadzu, Japan) by extracting pigments with N, N-dimethylformamide after keeping them in darkness for 24 h, and they were expressed in terms of fresh weight (FW) (Wellburn, 1994).

3. STATISTICS

Statistical Analysis Data were tested for normality with the Shapiro–Wilk test before statistical analyses. All results were presented as the mean ± SD of more than three replicates. Data were subjected to a one-way analysis of variance (ANOVA) with Tukey's posthoc test for mean separation. The t-test was performed where necessary. Bivariate analysis was used and followed by Pearson's correlation to evaluate the relationship among parameters. Statistical analyses were performed in IBM SPSS V25.

4. RESULTS

Figure 1 shows the H_2O_2 concentration in macrophyte tissues, as a function of temperature separately between different light intensity groups. The basal H_2O_2 concentrations were 4.6 µmol/gFW at 20° C for *E. densa* and *E. nuttallii*, and 3.0 µmol/gFW at 20°C for other species, respectively, after being exposed to dark conditions.

The H_2O_2 concentration changed with increasing temperature, such as −0.32 µmol/gFW/°C for *E. densa* (r = −0.985, $p < 0.01$), 0.39 µmol/gFW/°C for *M. spicatum* (r = 0.800, $p < 0.05$), 0.41 mol/gFW/°C for *C. demersum* (r = 0.900, $p < 0.01$), 0.60 µmol/gFW/°C for *P. crispus* (r = 0.974, $p < 0.01$), and 0.48 µmol/gFW/°C for *E. nuttallii* (r = 0.956, p < 0.01), respectively. For each species, H_2O_2 concentrations for different light intensity groups were plotted nearly in parallel, higher with higher light intensity groups (p <0.01). The tissue H_2O_2 concentration linearly increased responding to increasing water flow velocity for all these species (Figure 2). The increasing rate of H_2O_2 concentration with respect to flow velocity showed no significant difference among species with the gradient due to the velocity of 0.09 H_2O_2/velocity (µmol/ gFW/cm/s) (r = 0.921, $p < 0.01$ for *E. densa*, r = 0.878, $p < 0.01$ for *E.nuttallii*, r = 0.875, $p < 0.01$ for *P. crispus*, r = 0.700, $p < 0.01$ for *C. demersum* and r = 0.957, $p < 0.01$ for *M. spicatum*).

The laboratory results significantly agreed with field sample results, 0.072 H_2O_2/velocity (µmol/gFW/cm/s). In the field, no significant difference was obtained among the sampling seasons, and different rivers, if we remove the effect of temperature with the aid of Figure 1.

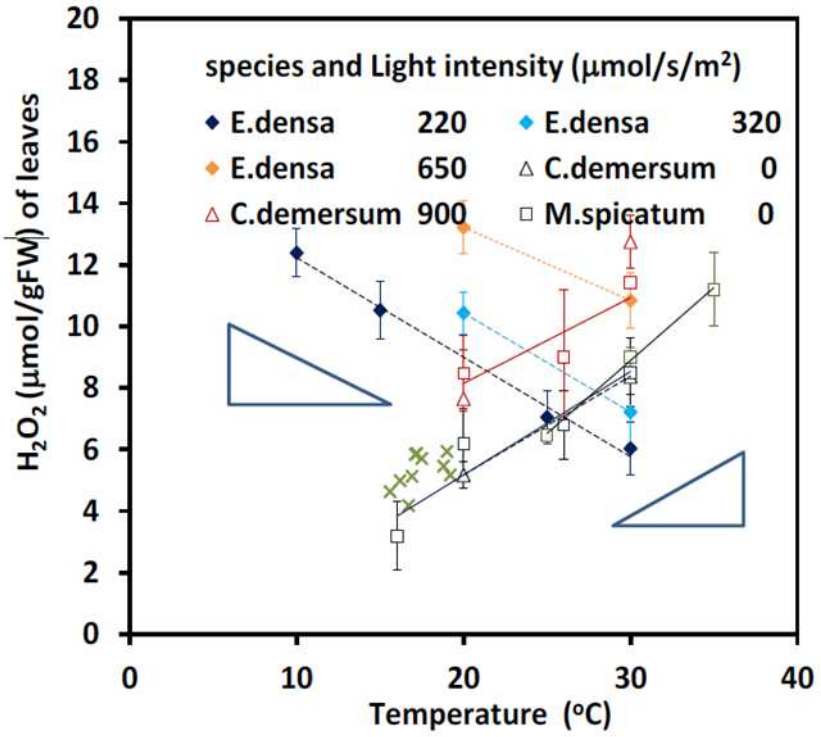

Figure 1. Temperature vs. leaf H_2O_2 concentration.

The increments of H_2O_2 concentrations for the light exposed samples with respect to the dark-adapted samples are shown in Figure 3. Experimental and field samples had a similar increasing trend. The increasing trend was obtained for all species, although the magnitude of the increasing rate was different between species, such as

$$H_2O_{r2.rad}(Temp) = \frac{1}{10}\left[I_0 e^{(-kz)} - 40\right]^{2/3}$$

for $I_0\,e^{(-kz)} \geq 40$ µmol/m²/s (1)

$$H_2O_{r2.rad}(Temp) = 0$$

for $I_0\,e^{(-kz)} < 40$ µmol/m²/s

for *E.densa* and *P.crispus*

$$H_2O_{r2.rad}(Temp) = \frac{1}{18}\left[I_0 e^{(-kz)} - 40\right]^{2/3}$$

for $I_0\,e^{(-kz)} \geq 40$ µmol/m²/s (2)

$$H_2O_{r2.rad}(Temp) = 0$$

for $I_0\,e^{(-kz)} < 40$ µmol/m²/s

for *C.demersum, M.spicatum, E.nuttallii*

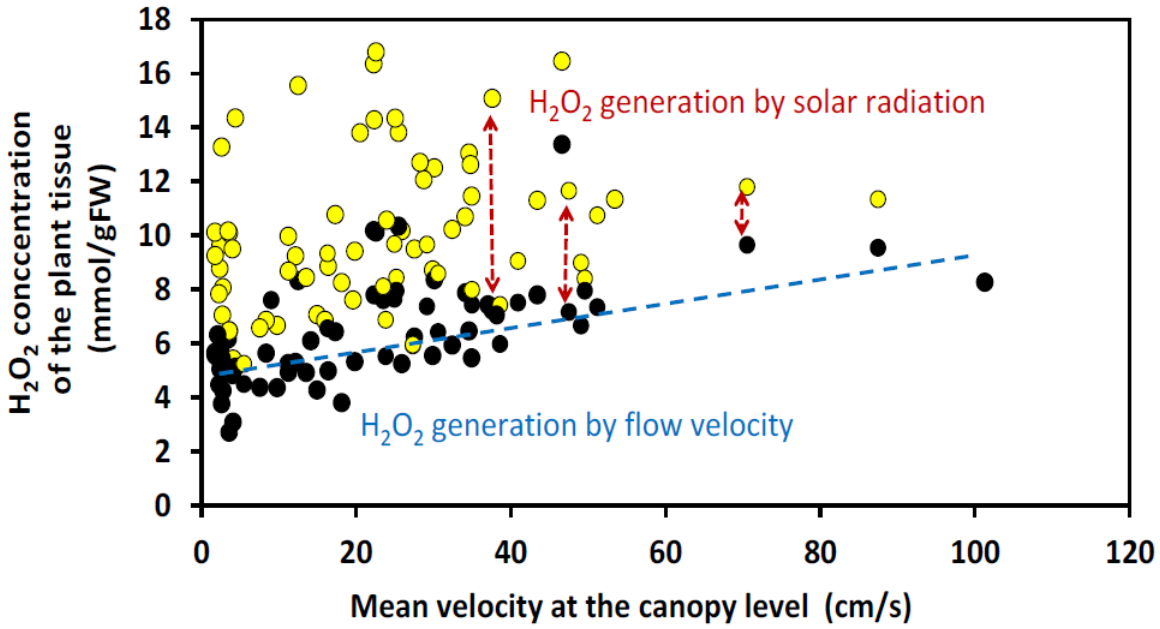

Figure 2. H_2O_2 concentration of leaf tissues with respect to mean velocity at canopy height, for light exposed and dark adapted samples.

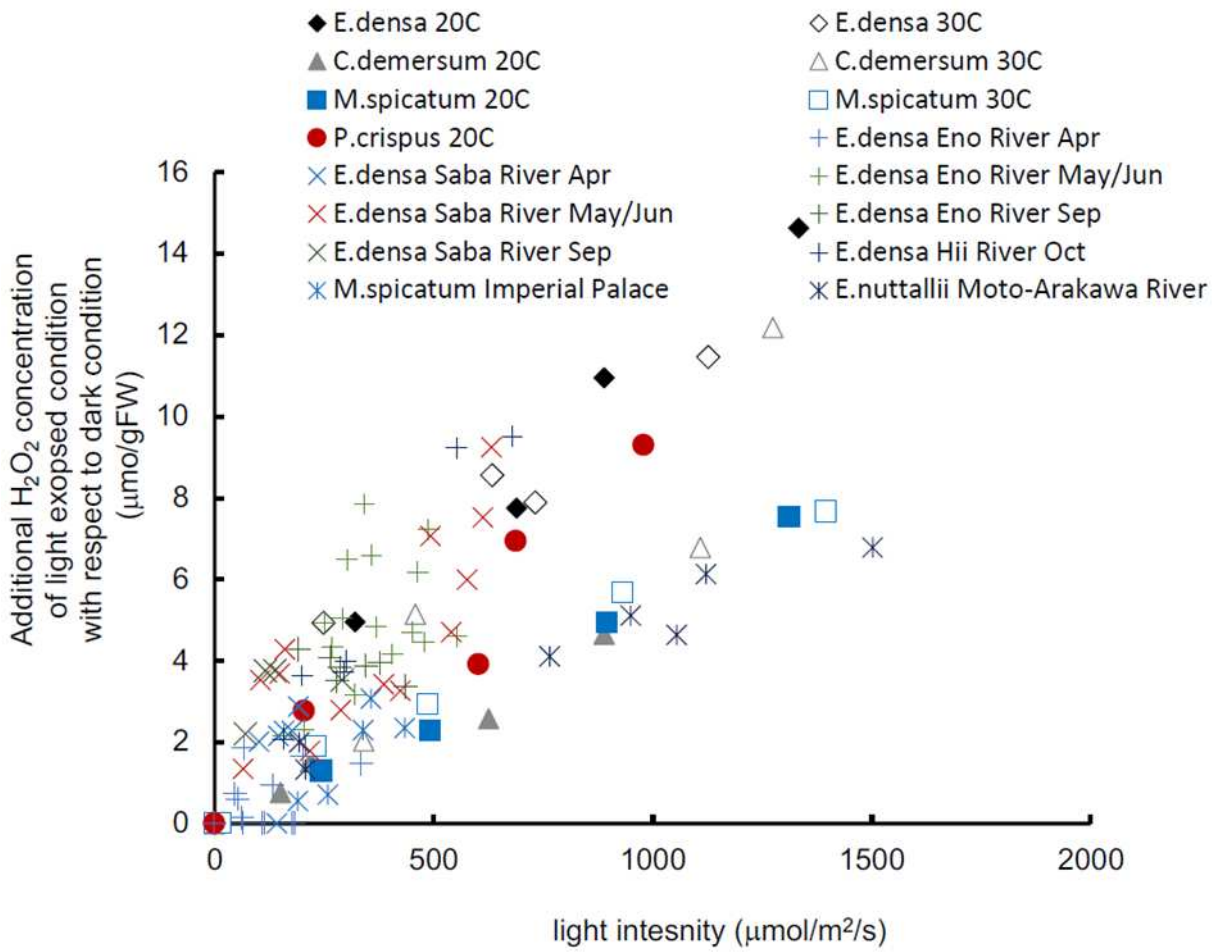

Figure 3. Increment of H_2O_2 concentration after exposed to light with respect to dark-adapted condition

Chl-a concentration and relative growth rate (RGR) obtained by the experiment decrease with increasing H_2O_2 concentration (Figure 4).

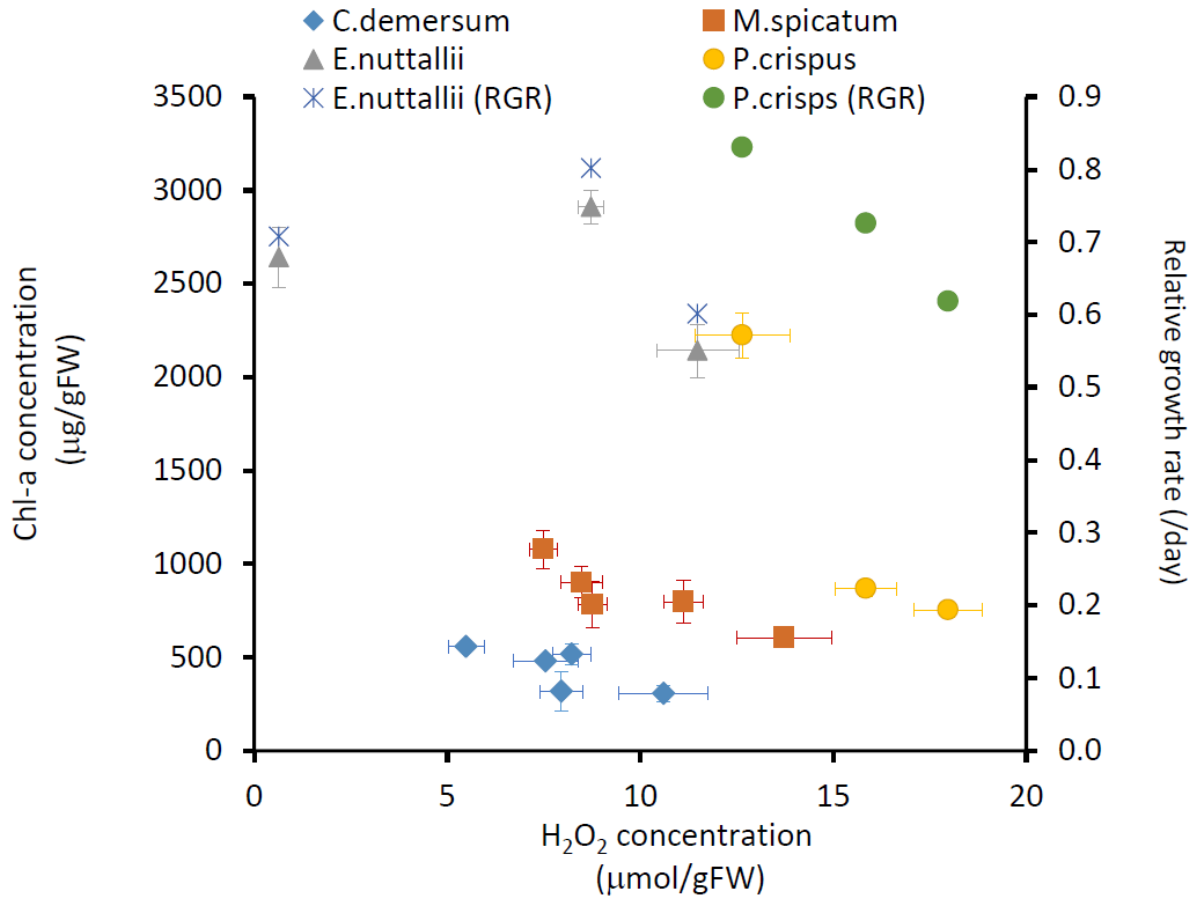

Figure 4. Chl-a concentration and relative growth rate as a function of H_2O_2 concentration

5. DISCUSSION

5.1 General trend of H_2O_2 concentration of tissues

There are several types of major stressors acting on submerged macrophytes in natural rivers, such as temperature, drag or fluctuating pressure associated with surrounding flow (Asaeda et al. 2020), water quality, solar radiation (Asaeda et al 2023), biotic stresses in the competition of other species (De Silva et al 2017), etc. The formation of colonies, growth rate of each plant, etc., are highly dependent on the tolerance against these environmental stresses.

In the management of these submerged macrophytes, or even to understand their physiological condition, it is extremely desirable to evaluate the degree of stress the plant s subject to, and particularly the share of each stress in the entire stress. However, these different types of abiotic stresses have different unit system to show their magnitude, and thus is difficult to compare directly (Asaeda et al 2021). A unified indicator is required.

When plants are subjected to unpreferable environmental conditions, due to different types of stress factors, ROS are generated in their body, associated with the degree of unfeasibility or stresses, in different organelles (Zaman and Asaeda, 2013; Das and Roychoudhury, 2014; Asaeda et al., 2017; Choudhury et al., 2017; Parveen et al., 2017; Asaeda et al., 2018), which then damages their cells by the oxidative stress. A part of ROS is scavenged quickly by antioxidants (Omar et al., 2012), and the homeostasis of the plant is maintained by balancing the ROS and antioxidants. The balance flips over when oxidative stress surpasses the scavenging capacity of the antioxidants (Dumont and Rivoal, 2019). Among ROS, hydrogen peroxide (H_2O_2) is widely generated (Asada, 2006; Sharma et al., 2012), in the process of scavenging ROS. Therefore, H_2O_2 has a possibility to be indicator of ROS activity.

As H_2O_2 is generated following each stress intensity, any type of stress is quantitatively evaluated by the H_2O_2 concentration. The comparison of intensity of different types of stresses becomes possible.

This study indicates that the amount of H_2O_2 increased parallelly following the intensity of a particular stress, as long as other stresses were same. The interaction between different type stresses seems to

be negligible. Therefore, once the relationship between the stress intensity and the H_2O_2 concentration is obtained stress by stress, we can estimate the amount of H_2O_2 concentration when multiple stresses are loaded as the sum of contributions of these stresses.

Chlorophyll -a concentration and biomass apparently decreased with increasing H_2O_2 concentration. When the H_2O_2 concentration exceeds some threshold value, the plant was extremely deteriorated and finally died out in several days. The H_2O_2 concentration is the tolerance level of the plant. The system is extremely useful to monitor the physiological condition of plants in short time, and this mechanism is available for any type of plants. In the field of ecohydraulics, although mechanical stresses are often focused, this system is available to understand the physiological condition of the plant (Paul and Kerpen 2022; Carus et al. 2022; Villanueva et al., 2022; Cornacchia 2023).

5.2 Threshold H_2O_2 concentration to die off plants

Figure 4 presents the relationships of H_2O_2 and Chl-a concentrations for *M. spicatum*, *C. demersum*, *E. nuttallii*, and *P. crispus.* Chl-a concentration decreased with the H_2O_2 concentration ($r = -0.896$, $p < 0.01$ for *M. spicatum*, $r = -0.752$, $p < 0.01$ for *C. demersum*, $r = -0.497$, $p < 0.01$ for *E. nuttallii*, and $r = -0.963$, $p < 0.01$ for *P. crispus*), and was eliminated at approximately 20 μmol/gFW. In the field observation, tissue as deteriorated when similar H_2O_2 concentrations continued for a few days. Both Chl-a concentration and RGR declined with increasing H_2O_2 concentrations in a similar way, regardless of different stresses, such as flow velocities, water temperatures, and light intensities (Chl-a concentrations and RGR decreased with increasing H_2O_2 concentrations (flow velocity $r = -0.944$, $p < 0.01$ for Chl-a and $r = -0.964$, $p < 0.01$ for RGR; temperature $r = -0.945$, $p < 0.01$ for Chl-a and $r = -0.980$, $p < 0.01$ for SGR; light $r = -0.924$, $p < 0.01$ for Chla and $r = -0.965$, $p < 0.01$ for RGR). This monitoring system has several possibilities applicable to ecohydraulics. The system is available to monitor the physiological/ecological condition of plants in a short time. In the field of ecohydraulics, mechanical stresses induced by drags are often focused (Paul and Kerpen 2022; Carus et al. 2022; Villanueva et al., 2022; Cornacchia 2023), this system is available to estimate the physiological/ecological condition of the plants, too. Subjected to stresses, ROS is produced with any type of plants. Therefore, the system is applicable to other types of plants, such as terrestrial plants, phytoplankton, etc., too., although some modification is required.

5.4.2 The period to exposure to stresses

In the present field observation, we made 30 min of dark condition before sampling macrophytes to get the results without high light stress. The reaction of oxidative stress is supposed to be extremely quick. But, there was some delay to get the stable condition. Figure 5 shows the ratio of the values with respect to values at the starting of darkness. The values slightly decreased until 30 min, then increased afterwards. The lowest values seem to be the dark-adapted values. However, the difference was only 20% until 6hrs. so, it is recommended to get samples before six hours after changing the condition, even if sampling at 30 min. is impossible.

5.4.3 Estimated error levels in the flume experiment

In the flume experiments, available velocity is normally less than 50cm/s. This velocity introduce approximately 5μmolH_2O_2/gFW. It corresponds to about 10°C difference in temperature change with respect to H_2O_2. Temperature fluctuation during experiment can be limited to 1-2°C in the indoor flume, Temperature fluctuation cannot be a large error. However, in case of outdoor flumes, temperature fluctuation is not negligible. 500μmol/m2/s of light intensity introduces about 5μmolH_2O_2/gFW of H_2O_2 concentration for *E. densa* and *P. crispus*, and 1000μmol/m²/s. This light intensity is introduced by sunlight from the windows, even in indoor flumes. A part of light exposure to a flume may affect the plant's condition. If the effect of flow velocity on the traits related to physiological conditions is targeted, the change in the ambient light intensity is limited to at most 100 to 200μmol/m²/s.

6. CONCLUSIONS

Aquatic plants face different types of abiotic stresses in nature, which affect the physiological condition of the plant, the growth or survival rates, and colony formation. Subjected to environmental stresses, hydrogen peroxide, H_2O_2, is generated in the plant cell, associated with the stress intensity and damages the plant body. The amount of H_2O_2 increased with increasing each stress intensity, and when multiple stresses are loaded, the total H_2O_2 concentration generated in the plant body is given by the sum of contribution of each stress. When the H_2O_2 concentration exceeds a threshold value, the plant was deteriorated and finally died out in several days. Therefor colonies cannot be formed, there. With this

indicator, the effect of different types of stresses can be compared separately, even if the quantification units are different. It will become a strong tool in ecohydraulics.

7. ACKNOWLEDGEMENTS
This work was financially supported by the Grant-in-Aid for Scientific Research (B) (19H02245) and (C)(23K04040), and Fund for the Promotion of Joint International Research (18KK0116) of Japan Society for the Promotion of Science (JSPS).

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