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HYDROGEN PEROXIDE CONCENTRATION AS AN INDICATOR OF ABIOTIC ENVIRONMENTAL STRESS OF MACROPHYTES FOR MANAGEMENT

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Abstract. Invasive species, *E. densa*, and Japanese native species, *Myriophyllum spicatum* and *Ceratophyllum demersum* were investigated in experimental tanks with different environmental conditions. Tissue hydrogen peroxide (H_2O_2) concentrations were measured responding to either individual or multiple environmental storessors of light intensity (PAR), and water temperature. The H_2O_2 concentration increased in parallel to the increment of unpreferable levels of each abiotic factor, and the trend was independent of other factors. The total H_2O_2 concentration is provided by the sum of contribution of each factor. Under increased total H_2O_2 concentration chlorophyll-a (Chl-a) concentration of plant tissues decreases, then reduce their growth rate, and subsequently reduce their biomass. The H_2O_2 concentration threshold, beyond which degradation is initiated, was between 16 and 20 µmol/gFW regardless of the type of environmental stresses. These results highlight the potential efficacy of total H_2O_2 concentration as a proxy for the overall environmental condition. In Japanese waters, major environmental factors limiting macrophyte colonization were identified as water temperature, and high solar radiation. The relationship between the unpreferable levels of these factors and H_2O_2 concentration was empirically obtained for these species. Then a mathematical model was developed to predict the colonization area of these species with environmental conditions. The tissue H_2O_2 concentration decreases with increasing temperature for *E. densa* while increases for other species. Therefore, native species grow intensively in spring; however, they often deteriorate in summer. For *E. densa*, on the other hand, H_2O_2 concentration decreases with high water temperature in summer, allowing intensive growth. High solar radiation, however, increases the H_2O_2 concentration, deteriorating the plant in shallow zones.

Keywords: submerged macrophytes, environmental stress, hydrogen peroxide, stress indicator, invasive macrophytes

1. INTRODUCTION

Submerged macrophyte response to environmental conditions is species specific, and invasive plants tend to exhibit more tolerance than native species (Bates et al., 2013). Therefore, invasive species are able to dominate or distribute in areas where native species fail to survive. Among different invasive aquatic macrophytes, *Egeria densa* is a well-known worldwide species that causes significant ecological issues in freshwater ecosystems. In Japan, E. densa was introduced as an ornamental aquarium plant in the early 19th century, however, it had escaped into natural freshwater bodies and became naturalized in the 1940s, then, was mainly invaded into lakes in the last two decades. Japanese lakes were nearly free of macrophytes in 1960s, however, they are now intensively covered, though the mechanisms are unknown.

The investigation of submerged macrophyte behaviors is normally conducted by a long term monitoring. However, it is a hard work and costly (Biggs, et al, 2019; Vettori and Rice, 2020).

Plants suffer from oxidative stress when exposed to harsh environments, which results in an enormous amount of reactive oxygen species (ROS) in cells (Sharma et al. 2012). Under normal conditions, the activities of reactive oxygen species are balanced with antioxidants generated as a defense mechanism. However, under stressful conditions, antioxidant defenses become insufficient, resulting in oxidative stress that often leads to cell death (Choudhuri et al. 2017). Among the different types of ROS, superoxide anion (O_2^-) and hydroxyl radicals (OH^-) are the dominant species generated under stress and are routinely converted into relatively stable hydrogen peroxide (H_2O_2), via either superoxide dismutase (SOD) or nonenzymatic systems (Asada 2006; Das et al. 2014). Thus, the concentration of H_2O_2 has a strong potential to provide information regarding the environmental stress imposed on plants (Asaeda et al. 2018; Asaeda et al. 2020).

The objective of the current study is to understand the species specific preferable condition for colonization along the riparian zone by quantifying environmental stress using hydrogen peroxide concentrations.

2. MATERIALS AND METHODS

2.1 EXPERIMENTAL PROCEDURE

An experiment was conducted to identify the increment of H_2O_2 concentration of the plant tissue under different water temperatures and irradiance levels (PAR), and, thereby, to make empirical relations between these factors. Several light levels (0–1,300 μmol/m^2/s of PAR) were tested in small aquaria (dimensions: 50.0 cm ×35.0 cm × 35.0 cm). Temperature level was maintained at 10 ± 2 (*E. densa*), 15 ± 2 (*E. densa*), 20 ± 2, 25 ± 2 (*E. densa*), and 30 ± 2, 35 ± 2 (*E.densa*)°C using a temperature controlling system (Aquarium cooler ZC-100α, Zensui Corporation, Tokyo, Japan). PAR intensity was irradiated under natural solar radiation or using LED lights (Model LT-NLD85L-HN, OHM Electric Inc., Japan) with a12 h light:12 h dark photoperiod for 3 weeks. The length of *E.densa* grown in the experimental units was measured using a millimeter scale at 5–7 day intervals. The shoot growth rate (SGR) was calculated as the difference in shoot length between two observations divided by the duration, and it was expressed in cm/day.

2.2 ANALYSIS

Fresh plant shoots were extracted (~500 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 TiSO4 method (Satterfield and Bonnell, 1955) with modifications. The reaction mixture contained 750 µl of enzyme extract and 2.5 ml of 1% TiSO4 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). The results were compared with those of the e-FOX method, and suitable agreement was obtained (Queval et al., 2008).

Chlorophyll 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; DeSilva and Asaeda 2017).

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 post-hoc 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.

3. RESULTS

3.1 Empirical relationships of H_2O_2 concentration with respect to abiotic factors

A species-specific response is shown on H_2O_2 formation in macrophyte tissues with the combined stresses of temperature and light intensity (Figure 1). The basal H_2O_2 concentrations were 4.6 µmol/gFW at 20°C for E. densa, and 3.0 µmol/gFW at 20°C for other species, respectively, after being exposed to dark conditions. The increment of H_2O_2 driven by the temperature change were −0.32 µmol/gFW/°C for *E. densa* ($r = -0.985$, $p < 0.01$), 0.39 µmol/gFW/°C for *M.spicatum* ($r = 0.800$, $p < 0.05$), and 0.41 µmol/gFW/°C for *C.demersum* ($r = 0.900$, $p < 0.01$), respectively. H_2O_2 concentrations of different light intensity groups were nearly in parallel, higher with higher light intensity groups ($p < 0.05$).

The increments of H_2O_2 concentrations for the light exposed samples with respect to the dark-adapted ones are denoted in Figure 2. PAR intensity had significant impacts on the H_2O_2 metabolism in macrophytes. For all species, H_2O_2 concentrations for different light intensity groups were plotted nearly in parallel, higher with higher PAR intensity groups ($p < 0.01$).

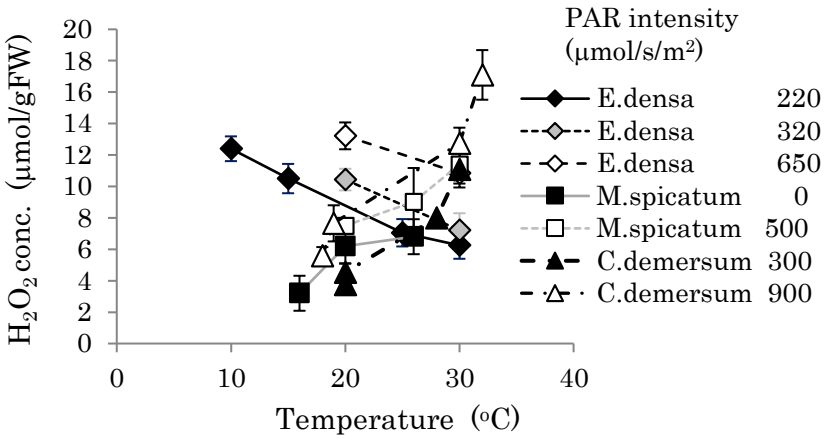


Figure 1. Tissue H_2O_2 concentration vs. water temperature

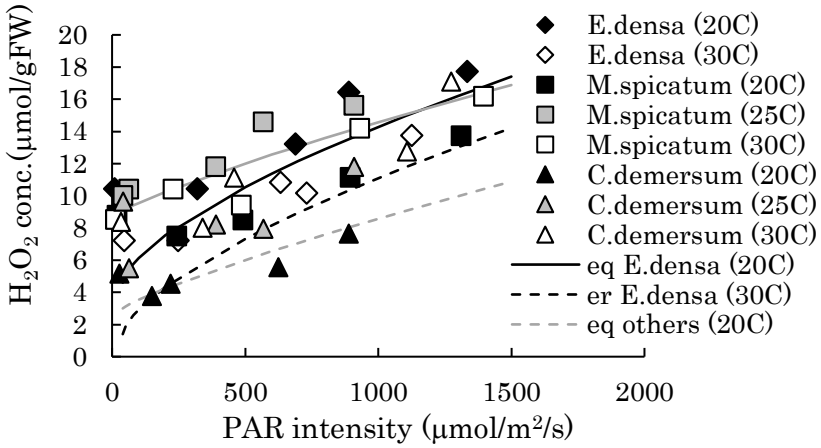


Figure 2. Tissue H2O2 concentration vs. PAR intensity (Equations are in MODELING)

These results indicate the independence of these stresses in the activity to generate H_2O_2 at least in practical level, suggesting the total value by the sum of H_2O_2 generated by each stress (Suzuki et al. 2014).

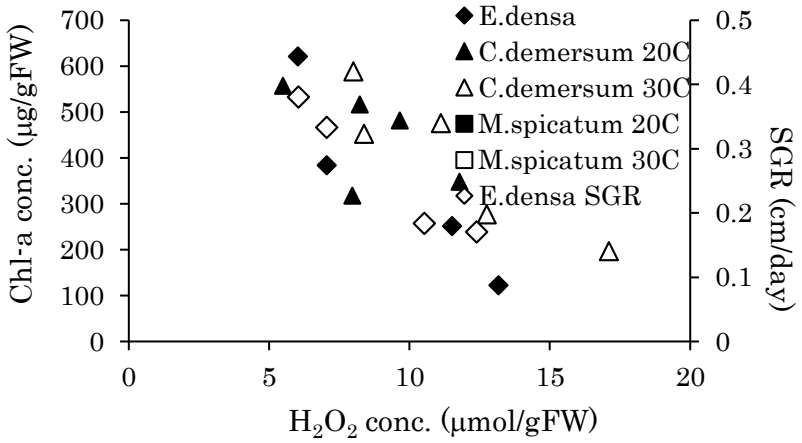


Figure 3. Chlorophyll- a concentration and SGR vs. tissue H_2O_2 concentration

4 MODELING

Figure 1 indicates that depending on species, H_2O_2 concentration nearly linearly increases or decreases with increasing temperature. Temperature dependence of H_2O_2 concentration is −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), and 0.41 μmol/gFW/°C for *C.demersum* (r=0.90, p < 0.01), respectively.

From Figure 2, the following relationships were obtained for the generation rate by PAR intensity:

$H_2O_{2\ rad}(Temp) = (I_0e(-kz) - 40)^{2/3}/10$ for ≥ 40 μmol/m²/s, *E.densa*

$H_2O_{2\ rad}(Temp) = (I_0e(-kz) - 40)^{5/6}/55$ for ≥ 40 μmol/m²/s, *M.picatum* and *C.demersum*.

Where I_o is the PAR intensity at the water surface (mmol/m²/s) and k is the attenuation coefficient of light intensity in water (/cm).

The total H_2O_2 concentration formed in plant tissues for a particular temperature (Temp) by the sum of H_2O_2 generated by metabolism (H_2O_{2met}), flow velocity (H_2O_{2vel}), and solar radiation (H_2O_{2rad}). If the value is between 16 and 20 μmol/gFW, then the growth of macrophytes deteriorates. Therefore,

$H_2O_{2tot}(Temp) = H_2O_{2rad}(Temp) + H_2O_{2met}(Temp) < H2O2_{cr}$ (15- 20 μmol/gFW)

assuming the effects of flow velocity (H_2O_{2vel}) and other factors such as low oxygen, low nutrients are relatively small (Ellawala et al, 2011; Parveen et al. 2019; Asaeda et al. 2020; Ranawakage and Asaeda 2020). Simulated total H_2O_2 concentration has high agreement with the observed values (Asaeda et al. 2020, 2021).

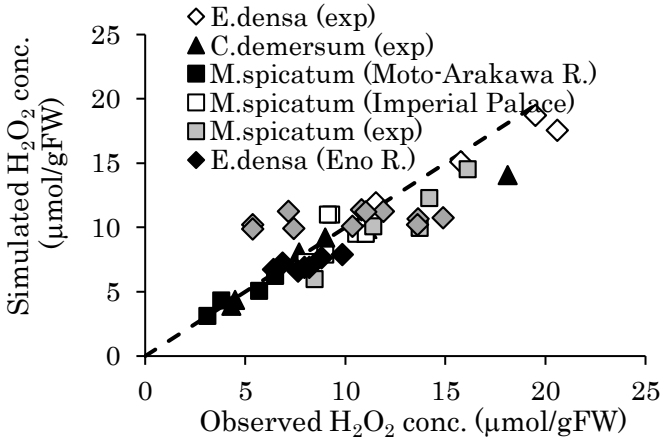


Figure 4. The comparison of total H_2O_2 concentration between simulation and observation

4. SIMULATED RESULTS AND DISCUSSION

Figures 5 and 6 denote the comparison between the simulated H_2O_2 concentration with the H_2O_2 concentration and biomass of experimental and observed results, compared with the threshold concentration, for *E.densa*, and *M.spicatum* and *C.demersum*, respectively.

Both the H_2O_2 concentration and the existing biomass range agreed well with observed data; H_2O_2 concentration: within 2.5 μmol/gFW. All positive biomass range was in the range where the H_2O_2 values were below the threshold, 16-20mmol/gFW. The H_2O_2concentration of *E.densa* exceeds the threshold with high PAR (Asaeda et al. 2020), thus it cannot colonize at shallow zones, while those of *M.spicatum* and *C.demersum* increases with increasing temperature, indicating the deterioration in high summer.

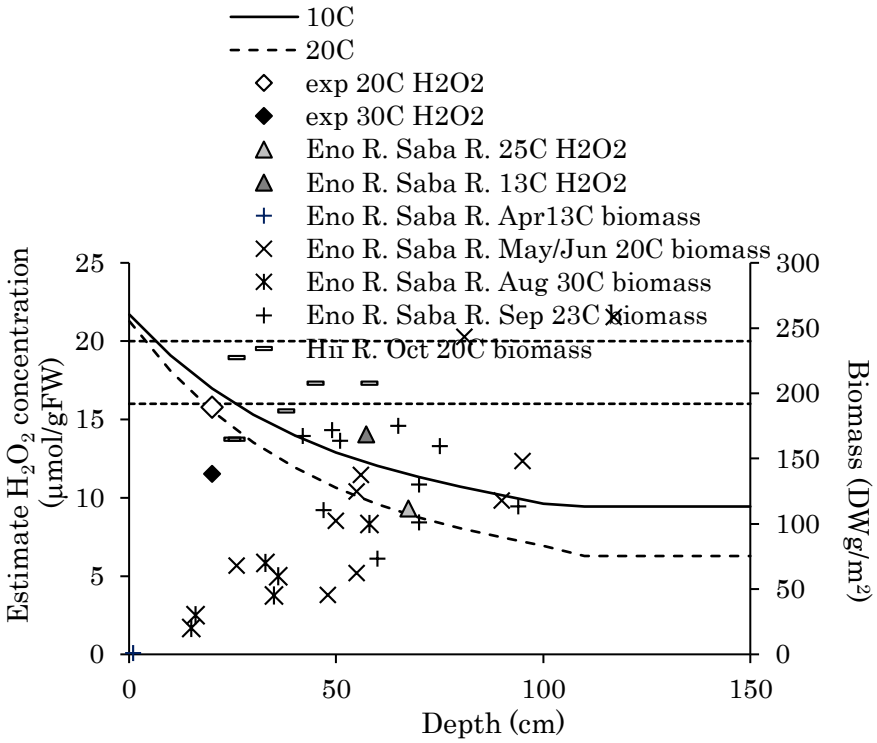
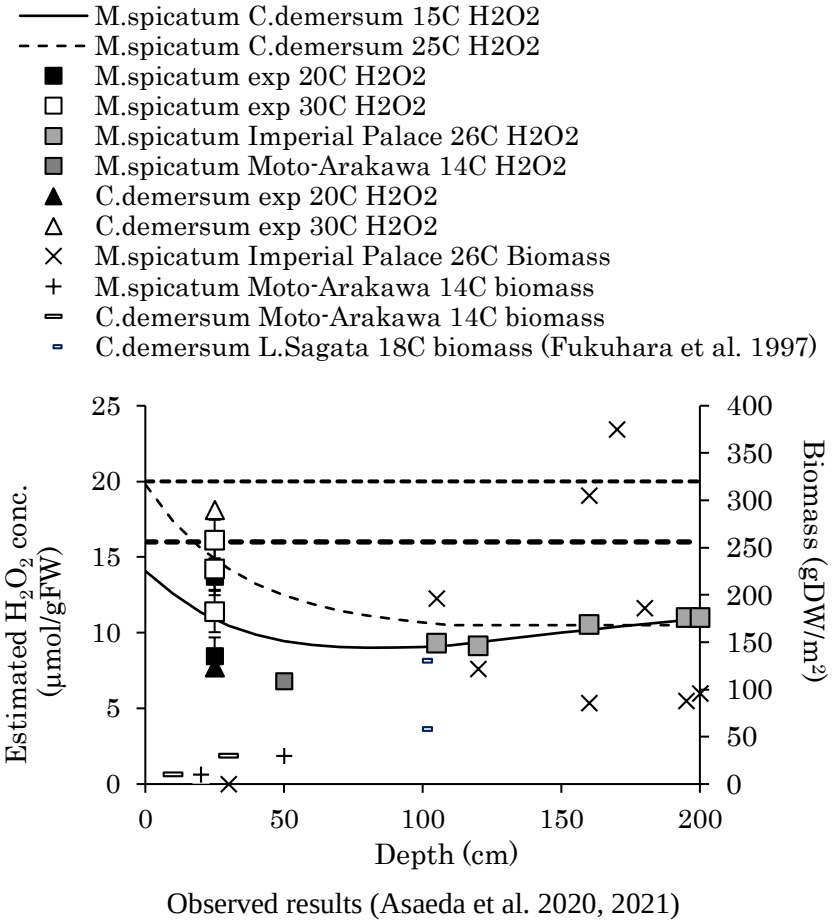


Figure 5. Estimated H_2O_2 concentration vs. observed H_2O_2 concentration and biomass for *E.densa*

Horizontal dashed lines show the threshold H_2O_2 concentration range and observed results are (Asaeda et al. 2020, 2021).

5. CONCLUSIONS

Under unpreferable environmental conditions, H_2O_2 concentrations increase in plant tissues and reflect the macrophyte condition fairly accurately. Potentially, this could be a good indicator of submerged macrophyte colonization. This approach will save time by not requiring casual observations and biomass monitoring of macrophytes in ecosystem monitoring. The experimental and field observations indicated a clear positive relationship between the level of unpreferable conditions and H_2O_2 concentrations, regardless of abiotic factors. The total H_2O_2 concentration is provided by the sum of H_2O_2 generated by each environmental factor, and <16–20 μmol/gFW is required for colonization. The relationships of H_2O_2 concentrations and the contribution of each abiotic factor were obtained for invasive species, *E. densa*, and three major Japanese native species, *M. spicatum*, and *C. demersum*. The system was applied to develop a mathematical model to simulate the colonization area of these species. The tissue H_2O_2 concentration decreases with increasing temperature for *E.densa* and increases for other species. Therefore, native species grow intensively in spring; however, they often deteriorate in summer. For *E. densa*, on the other hand, H_2O_2 concentration decreases with high water temperatures in summer, allowing intensive growth. High solar radiation increases the H_2O_2 concentration, deteriorating the plant. Currently, river rehabilitation has created a deep zone in the channel, which has supported the growth and spread of *E. densa*.



Observed results (Asaeda et al. 2020, 2021)

Figure 6. Estimated H_2O_2 concentration vs. observed H_2O_2 concentration and biomass for *M.spicatum* and *C.demersum*

Horizontal dashed lines show the threshold H_2O_2 concentration range and observed results are (Asaeda et al. 2020, 2021).

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CONFLICT OF INTEREST

Author TA was employed by Hydro Technology Institute of Japan. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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