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BRIEF COMMUNICATION

Effects of Epiphytic Load on the Photosynthetic Performance of a Seagrass, *Zostera marina*, Monitored In Vivo by Chlorophyll Fluorescence Imaging

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Abstract We investigated the effects of epiphytes on photosynthetic activity in a seagrass, *Zostera marina*. Parameters in our chlorophyll (Chl) fluorescence imaging technique, including Fo, Fm, and Fv/Fm, were monitored from leaf surfaces before and after those epiphytes were removed. Because of the uneven distribution of light intensities, Fm values at the margin of an image were underestimated while those in the central region were overestimated. Chl fluorescence emissions from all leaves except the youngest one were altered by the presence of epiphytes, which predominantly inhabited the surfaces of older leaves. Only a few were found lower on the plant where leaves were very close to each other. Regions where the epiphytes had been loosely bound before their gentle removal showed full restoration of photosynthetic performance to control levels afterward. However, only minor recovery of photosynthesis was found in areas that had been riddled with tightly bound epiphytes and were permanently damaged. In years 2002 and 2003, leaf productivity peaked in May and plummeted in November. More epiphytic diatoms were distributed when the seagrass biomass was larger, with pinnate diatoms dominating.

Keywords Chlorophyll fluorescence imaging · Epiphytes · Photosynthesis · Seagrass · *Zostera marina*

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Introduction

Seagrasses are annual or perennial species that grow along coasts and estuaries. These angiosperms differ from algae in having true leaves, roots, stems, and flowers. The seagrass community or "seagrass meadow" comprises very productive underwater vegetation that provides habitat for many other plant and animal species (Moncreiff et al. 1992). Because of this, such communities are quite vulnerable to human activities (Chung 2003). Seagrass growth is also limited by available light, a major factor in the submerged environment. In particular, epiphytic fauna and flora become attached to leaf surfaces and hamper photosynthesis by intercepting that light (Halin 1980).

Chlorophyll (Chl) fluorescence provides very useful information about the physiological status of photosynthetic organs or cells, acting as a nondestructive, noninvasive, and highly sensitive probe. A traditional Chl fluorometer has a single light detector that measures fluorescence intensity and can monitor the average value of signals from all cells within the area of an organ being sensed by the probe. In contrast, the same information about a leaf surface can be captured in vivo with

a CCD camera in a Chl fluorescence imaging system, which contains a two-dimensional array of light detectors. Therefore, such imaging is used for studying the heterogeneity of photosynthesis and monitoring localized infections by viruses and other pathogens before symptoms become visible (Balachandran et al. 1994; Ning et al. 1995; Scholes and Rolfe 1996; Osmond et al. 1998). This technique can also be applied to examining the local effects of irradiance, temperature, heavy metal stress, and perturbations of metabolism and plant growth (Genty and Meyer 1995; Siebke and Weis 1995; Lichtenthaler et al. 1996, 2000; Oxborough and Baker 1997; Buschmann et al. 2000; Langsdorf et al. 2000; Nedbal et al. 2000; Lee et al. 2001; Baker and Rosenqvist 2004; Kim et al. 2006). As a noninvasive high-throughput tool, its usage has been broadened to screen out specific mutants from libraries in order to disclose photosynthesis-related phenotypic effects of those mutations and to select homozygous pure lines from a pool of heterozygous offspring (Niyogi et al. 1998). In this study, we used Chl fluorescence imaging to investigate the influence of epiphytes on the photosynthetic activity of seagrass leaf cells, taking into account leaf age and location, as well as seasonal variations.

Materials and Methods

Materials

Seagrass (*Zostera marina*) was sampled from a sublittoral zone of Daguri in Jindong Bay on the southern coast of the Korean Peninsula in mid-October 2003. Approximately five leaves per plant were ordered by age, with the youngest labeled as the first. Each leaf was cut into five to nine 10-cm segments, which were arranged in parallel (left to right) on a plate prior to capturing its Chl fluorescence image (Fig. 1). To minimize drought stress during this preparation, the materials were placed on wet paper tissues and frequently sprayed with seawater filtered through Whatman GF/C paper.

Chlorophyll Fluorescence Image Analysis

Chl fluorescence images were taken with an imaging fluorometer (FluorCAM 700MF; P.S. Instruments, Brno, Czech Republic) according to the manufacturer's instructions. To measure in vivo slow fluorescence induction kinetics, the fluorometer was operated according to a standard protocol template for quenching analysis included in the operating software. Its light was modified to 50% sensitivity and 30% actinic light intensity (about 100 μmol photons m^{-2} s^{-1}). Images corresponding to Fo and Fm were then processed to obtain Fv/Fm images. Fo represented the basal Chl fluorescence level in a dark-adapted leaf, and Fm was the maximum fluorescence level emitted from a dark-adapted leaf that was exposed to a saturating light pulse. Fv indicated the maximum variable fluorescence, equal to (Fm−Fo). Fv/Fm was used to estimate the potential quantum yield, i.e., photochemical efficiency, of photosystem II (PSII) (Oh et al. 2001). Before these images were taken, all seagrass samples were dark-adapted for 10 min at room temperature.

Cell Counting

Epiphytic diatom samples were collected from the third leaves. Up to 11 segments were excised from each leaf tip at regular 10-cm intervals. The diatom cells were scratched from the leaf surfaces and collected with the filtered seawater. These were washed several times with seawater, concentrated to 10 mL, and preserved in Lugol's solution. The cells were tallied on a Zeiss Axioskope microscope (Zeiss, Germany) with a Sedwick-Rafter counting chamber. Afterward, they were fixed with 1% glutaraldehyde solution for species identification at the microscope.

Results and Discussion

To monitor the effect of epiphyte perturbations on the photosynthetic activity of seagrass, we measured three Chl

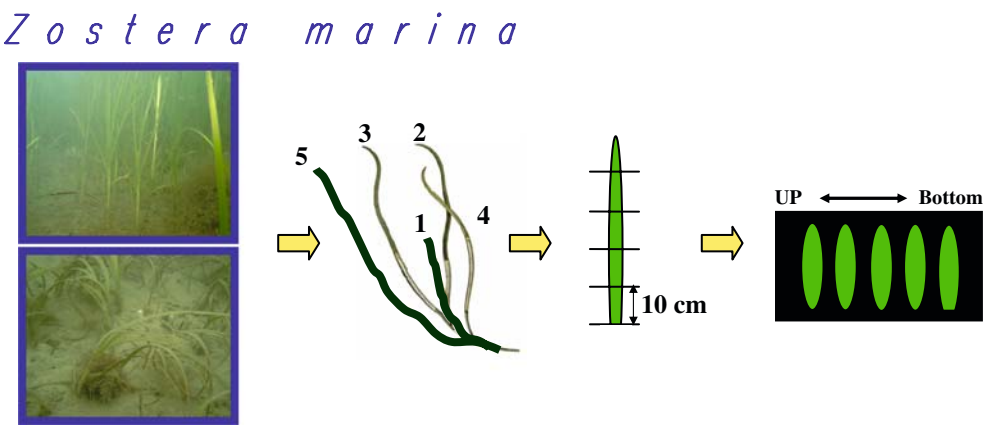

Fig. 1 Photo of *Z. marina* under water, and schematic diagram of sample preparation. Leaf order was assigned based on age and position (top/youngest=1). Segments (10 cm long) were made from tip of leaf downward and arranged *left* to *right* on plate for chlorophyll fluorescence imaging

fluorescence parameters—Fo, Fm, and Fv/Fm. Leaf images were taken twice, before and after the epiphytes were removed.

Fluorescence intensities (Fo) were evenly distributed in the first and second leaves (Fig. 2a, left). In these young organs, the epiphytes might have had little opportunity to colonize the leaf surface. However, the remaining leaves (third, fourth, and fifth) were occupied by many black spots where epiphytes were attached, with the older ones having more spots. For these leaves, the first five segments (from the tip), corresponding to the upper 50 cm, had more epiphytes than the lower portion. Because of the close spacing of the leaves on each plant, very few epiphytes settled on the lower segments, i.e., more than 80–90 cm down from the tip (data not shown).

The Fm image in Fig. 2a (middle) confirmed our Fo results because signals for both Fm and Fo were roughly proportional to Chl contents and the Fm signals produced from healthy leaves were generally five times stronger than those for Fo. Therefore, we could locate epiphyte positions more clearly in our Fm images. Intensities of the first and last leaf segments (Fig. 2b) were rather low, probably due to the uneven distribution of light from the saturating beam across the plates.

The photochemical efficiency of PSII, Fv/Fm, was not proportional to Chl content. In addition to its physical meaning, the use of Fv/Fm values rather than those of Fm or Fo to represent Chl fluorescence might alleviate such problems associated with that uneven distribution because both the denominator and the nominator carry the factors that are proportional to the light intensity of the saturation beam.

From the Fv/Fm image (Fig. 2a, right), we found that the epiphytes predominantly inhabited the surfaces of older leaves while few were found at the bottom of each leaf because of its proximity to others. Photosynthetic efficiency was mostly depressed where Fo and Fm were low. However, for the second leaves, we noticed a marked decline in the level of Fv/Fm in their upper portions, although such an observation was not consistent with the Chl fluorescence image that manifested Fm. This again was probably because of uneven distribution of light intensity that had been used for inducing fluorescence. Our results indicated that all leaves except the first (i.e., the youngest) were under the undesirable influence of epiphytes, as exemplified by diminished photosynthetic efficiency. Moreover, the extent of epiphytic attachment depended on leaf age, i.e., positioning, with epiphytes more likely to occupy the upper part of the blades where frequent contact was made with plankton floating in the water current.

Figure 2b shows images of Chl a fluorescence after epiphytes were gently removed. These images were clear and showed no black spots, indicating that some locations with previously low signals had recovered. This recovery

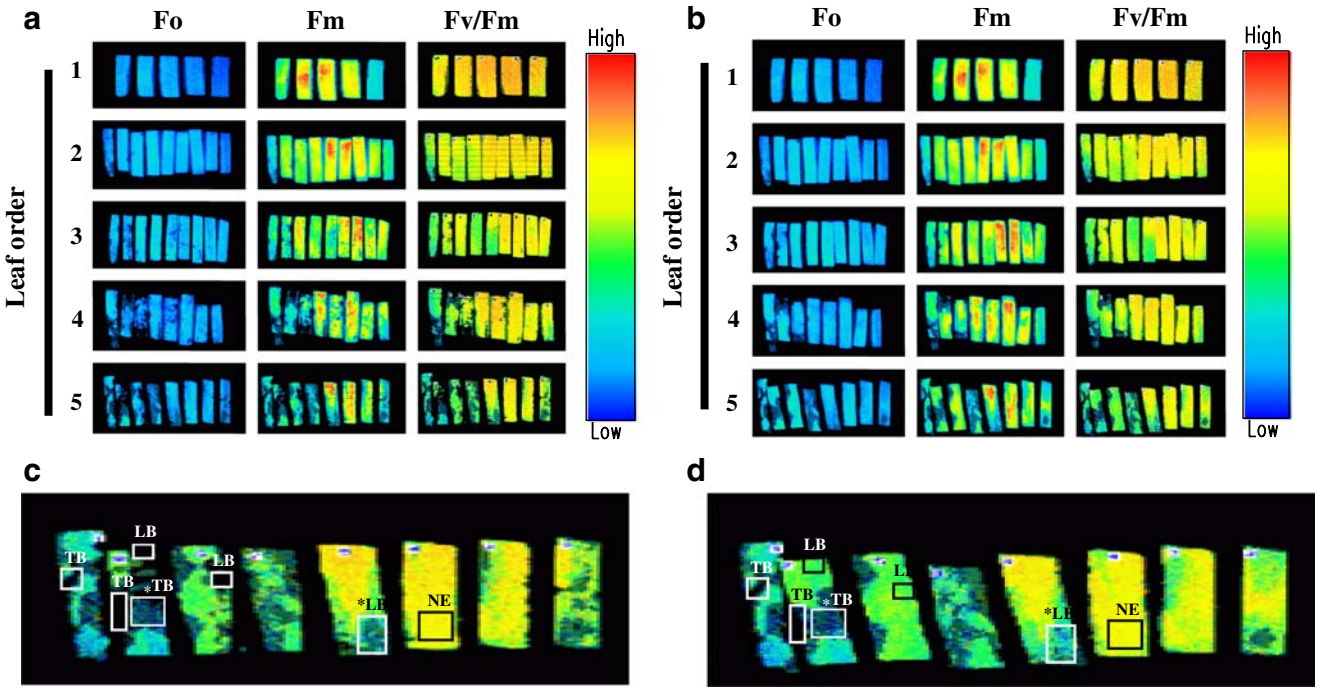

Fig. 2 Chlorophyll fluorescence images for Fo, Fm, and Fv/Fm in seagrass leaf segments before (**a**) and after (**b**) removal of epiphytes. Expanded chlorophyll fluorescence images for Fv/Fm of fifth leaf showing sectors with loosely bound (*LB*) and tightly bound (*TB*) epiphytes before (**c**) and after (**d**) removal of epiphytes. *NE* is sample sector without epiphytes. *Small photos* are images of segments on plates for imaging; each is colored on relative scale based on fluorescence intensity

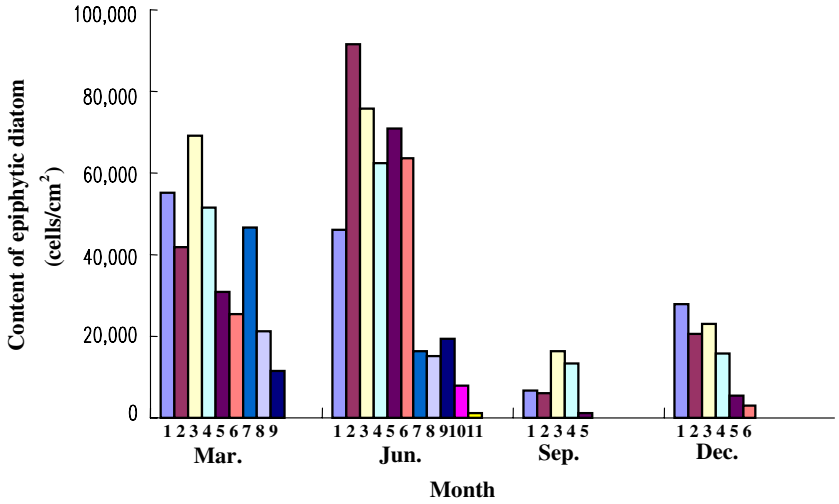

Fig. 3 Seasonal variation in amount of epiphytic diatom cells on third leaf of seagrass. *Number below each bar* indicates position of segment from tip of leaf

was more apparent in some areas of the third leaf that earlier had depressed Fm levels prior to cleaning. Likewise, in places where Fm had been partly recovered, photosynthetic efficiencies were somewhat improved. Elsewhere, however, even epiphyte removal did not restore values to control levels for either Fm or Fv/Fm. Nevertheless, the fourth and fifth leaves had regions with varying degrees of recovery.

Recovery was much more evident in the upper leaf portions as well as in younger leaves. This was more clearly seen in the expanded image of Fv/Fm (Fig. 2c, d), which separated both the recovered and the unrecovered sectors based on changes in photosynthetic activity after epiphytes were removed. Thus, it was possible to discriminate two different regions of leaves with different responses to removal. That is, the "loosely bound epiphyte sector" easily recovered its photosynthetic ability from epiphyte-influenced inhibition, whereas the "tightly bound epiphyte sector" was unable to do so. Although some cells were damaged when the epiphytes were scraped away, due to particulate detritus mixes, this susceptibility was very low for cells in the "loosely bound" sector because epiphyte removal was as gentle as possible. Fv/Fm values from the "tightly bound" epiphyte regions declined markedly, with images that were darker than in other regions. This implied permanent cell damage caused by the parasitic nature of those epiphytes.

We also examined seasonal changes in the biomass of epiphytic diatoms that inhabited the third leaves (Fig. 3). The number of epiphytic cells varied quite significantly over the year, ranging in 2003 from approximately 469,000 cells cm^{-2} in June to approximately 44,000 cells cm^{-2} in September. We previously reported similar seasonal variations in seagrass leaf elongation and production rates from March 2002 to December 2003 in Jindong Bay, on the southern coastal area of Korea (Lee et al. 2004). Our current study showed maximum leaf productivity in May, equivalent to 30.0 mg dw sht^{-1} day^{-1} (3.7 g dw m^{-2} day^{-1}) in 2002 and 20.0 mg dw sht^{-1} day^{-1} (2.2 g dw m^{-2} day^{-1}) in 2003. Productivity was lowest in November, i.e., 3.2 mg dw sht^{-1} day^{-1} (0.12 g dw m^{-2} day^{-1}) in 2002 and 5 mg dw sht^{-1} day^{-1}(0.12 g dw m^{-2} day^{-1}) in 2003.

Pinnate diatoms were dominant, with their appearance varying among seasons—March 2003: *Cocconeis scutellum* (50.8%), *C. placentula* (35.6%); June 2003: *C. scutellum* (34.7%), *Cymbella turgidula* (18.8%); September 2003: *Rhizosolenia delicatula* (29.1%); and December 2003: *Cocconeis scutellum* (48.0%), *C. placentula* (22.3%).

Species diversity was highest in June (data not shown). These observations indicated that changes in epiphytic biomass strongly depended on those of the seagrass, with marked increases occurring between Spring and Summer.

In summary, we have demonstrated that heavy attachment of epiphytic algae on the leaf surfaces of seagrass induces a loss of Chl fluorescence signals. Such disruption of photosynthesis in older leaves is associated with tightly bound epiphytic cells. Because those epiphytes are mixed with detritus, they intercept light and hamper photosynthesis, possibly leading to permanent leaf damage after prolonged contact. This can explain why no Chl fluorescence signals were detected in epiphyte-covered regions, even when epiphytic diatoms, including photosynthetically active epiphytic algae, were dominant (data not shown). Human activity is a major factor that influences the amounts of epiphytes and detritus. Based on these observations, we recommend the Chl fluorescence imaging technique as a useful research tool for analyzing the various interactions between seagrass and epiphytes in terms of their photosynthetic performance and primary production.

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