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Epiphytic effects of *Licmophora paradoxa* on pigments of *Pyropia yezoensis*

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Epiphytic diatoms usually cause adverse effects on photosynthesis of the host plants due to shading light or by interfering with the biochemical pathways. The present study investigated the epiphytic effects of the diatom *Licmophora paradoxa* on the pigments of red alga *Pyropia yezoensis*, such as the chlorophyll-*a* (Chl-*a*), Phycoerythrin (PE), Phycocyanin (PC), Allophycocyanin (APC) and carotenoid contents. The results showed that Chl-*a* was significantly decreased while other pigments such as PE, PC, APC and carotenoid contents were significantly increased in *P. yezoensis* due to the attachment of epiphytic diatom *L. paradoxa*. The present results indicated that epiphytic diatoms produced negative effects on the host PSII reaction center by reducing its main pigment Chl-*a*. Whereas, antenna pigments of Phycobiliprotein, of *P. yezoensis* such as PE, PC and APC were increased to capture more light energy supplying photosynthesis. The increase in carotenoid content under this epiphytic situation implied an enhancement of its assisted function in light-harvesting, photoprotection and stress-tolerance mechanism. The present findings contribute to well understanding the response mechanism of host macroalgae to epiphytic microalgae.

[**Keywords**: Epiphytic diatom, Epiphytic effects, *Licmophora paradoxa*, Pigments, *Pyropia yezoensis*]

Introduction

An epiphytic coating on the leaf or the algal frond can intrude for the uptake of carbon, slow down the photosynthesis and reduce the oxygen rate[1,2]. Heavy epiphytic masses on the leaf surface have been thought as the inducement of dieback and decreasing the production ability of seagrass *Potamogeton perfoliatus*[3]. Several other studies have reported that effects of epiphytes could produce change in the photosynthesis and membrane compounds of macrophytes and macroalgae[1,4-7]. Epiphytes reduce and indirectly influence the growth of host algae by sticking on its surfaces, or by creating competition for the nutrients and essential gases[2,8-9]. They also reduce the production and reproduction of the macrophytes by shading the light required for the host organisms[10-12]. The submerged macrophytes can be negatively influenced due to the variations in the photosynthetic process, epiphytic algae and low light phenomena both decrease the growth and production of the immersed macrophytes[2,3,13].

The existing exteriors of the marine climates are usually occupied over diversity of entities recognized such as epiphytes[8], which includes bacteria, diatoms, fungi, protozoans and sometimes by the algal spores[14], may adversely affects the host. For example, epiphytes can reduce the growth and may be the source of mechanical pressure to the aquatic macro organisms, like seagrass[15-16]. Kim *et al.*[9] reported that vast concentrations of epiphytes such as diatoms may cause negative influences on the production of *P. yezoensis*. It was domenstrated that effects of epipytic diatom *Licmophora paradoxa* can seriously affect the physiological indexes of *Pyropia yezoensis*, such as Malondialdehyde (MDA) content and antioxidant enzymes Superoxide Dismutase (SOD), and Catalase (CAT), which are the internal stress indicators were significantly increased. Similarly, the photosynthetic measurements of maximum quantum efficiency of PSII (Fv/Fm), the maximum electron transport rate (rETRmax), the minimum saturating irradiance (Ek) were significantly decreased in *P. yezoensis*, due to previous attachment of diatoms on its surface[7].

Pyropia yezoensis (Ueda) M. S. Hwang & H. G. Choi is a marine macro alga, belonging to class Bangiophyceae, order Bangiales, family Bangiaceae[17]. The alga is commercially important throughout the world, with the production of

1.8 million metric tons annually and with a high economic value[18]. Similar to other marine macroalgae, *P. yezoensis* also provides its surface to different kinds of epiphytes, which may be pathogenic or non-pathogenic to its survival[9]. Epiphytic diatoms are one of the major negative effect causing agents to the quality of *P. yezoensis.* Pennate diatoms such as *Licmophora* spp. are the abundant agents occur, producing heavy loss on the production of *Porphyra* spp.[19]. Epiphytic diatoms can adversely affect the growth of *P. yezoensis*, because of competion for nutrients and causing bleaching of macroalgal thalli. It may affect the odor as well as the purity and quality of the infected *P. yezoensis*[9]. Shading the light for *P. yezoensis* can seriously affect its growth as it is directly related to its photosynthetic efficiency[7].

P. yezoensis includes different kinds of pigments, which are needed directly or indirectly for photosynthesis. Among them, chlorophyll pigments are the main photosynthetic green pigments and epiphytic organisms may produce such applications to bring damaging effects on photosynthetic organelles thus reducing the concentration of chlorophyll-*a* (Chl-*a*) of macrophytes[3,4,6]. Similarly, carotenoids are yellow, red or orange photoprotective pigments that pass the absorbed energy to chlorophyll[20] and the main photosynthetic accessory pigments in cyanobacteria, rhodophytes, cryptomonads and cyanelles are phycobiliprotiens, which are endosymbiotic plastid-like organelles[21-22]. Phycobiliproteins contribute in a very effective energy transfer chain in the reaction centers of PSII which are liable for around 50 % of light taken in the cyanobacteria and rhodophytes[22]. It is reported that marine macroalgae *Kappaphycus alvarezii* covered with epiphytes tend to adapt the situation to the little light circumstances through increasing its photosynthetic pigments, particularly phycobiliprotein[23]. Thus, the pigments play a significant part in the development and replica of macroalgae.

To date, many scientists have investigated the community structure dynamics of epiphytism on macroalgae; however, few have focused on the negative effects and extent of harm to macro algae. The present study aimed to determine the negative influence of the epiphytic diatom *L. paradoxa* over the Chl-*a*, Phycoerythrin (PE), Phycocyanin (PC), Allophycocyanin (APC) and carotenoid contents of *P. yezoensis*.

Materials and Methods

Sample preparation

P. yezoensis was cultured at 10 °C following the standard laboratory protocol using Provasoli's enriched seawater medium culture as a growth medium[24]. On the other hand, F/2 medium was used to culture the diatom *L. paradoxa* separately at 20 °C[25-26]. To make a co-culture system for diatoms and the *P. yezoensis*, small pieces of about 2 cm^2 area of *P. yezoensis* were taken in a 1 L bottle having 10.4×10^5 cells ml^{-1} of diatoms cells using F/2 growth medium and labelled as the treatment bottle. Similarly, same protocol was followed to culture *P. yezoensis* without diatom cells, which was labelled as the control bottle. The cultures were exposed to the light intensity of 65 μmol m^{-2} s^{-1} with 12/12 hours of day and night sequence at 15 °C. After 9 days of co-culturing, the treatment bottle was taken and the diatoms cells were removed using a soft silicon brush. The experiments were performed in triplicates. Finally, Olympus BX53 microscope (Olympus, Japan) was used for the confirmation of successful removal of diatoms from the superficial of *P. yezoensis.* The treatment and the control samples were kept at − 20 °C for more experiments.

Pigment measurements

Determination of chlorophyll-a

Chl-*a* content of the treatment and control samples was extracted by means of 0.5 g in acetone (90 %) solution for 48 h at 4 °C in the dark. The extractions were centrifuged at the 4000 rpm for 10 min at 4 °C and the supernatants were used to conclude the absorbance via a spectrophotometer at the wavelengths of 750 nm, 664 nm, 647 nm, and 630 nm, correspondingly. The Chl-*a* content was determined and calculated via the subsequent equation:

$$\text{Chl-}a = 11.85_{E664} - 1.54_{E647} - 0.08_{E630}\ ^{(\text{ref. 27})} \quad \ldots (1)$$

Determination of phycobiliprotein

The determination of phycobiliprotein content was followed using the method of Hongfeng[28] with slight modifications. An accurate weigh of 0.010 g of the control and the treatment algal samples were taken and dried in an oven at 80 °C up to 6 h. An appropriate amount of cleaned water was added to a mortar, and grinded to make a homogenate. Thereafter, the slurry was transferred in to a 5 mL of a centrifuge tube, and after repeated freezing-ablation for several times, stored at - 20 °C.

The extracts of samples were substantially dissolved in the water. Thereafter, the mixture was centrifuged (8000 rpm, 10 °C, 15 min), the supernatant was taken, and were determined at the wavelengths of 565, 615 and 652 nm of spectrophotometer for determination of Phycoerythrin (PE).

The below formula was used for determination of PE in which V represents the capacity of supernatants and the W represents the dehydrated weight of the samples. The OD value was substituted into the following formula to calculate PE, PC and APC content (mg/g).

$$PE = (0.123 \times OD565 - 0.07 \times OD615 + 0.015 \times OD652) \times V / W \quad \ldots (2)$$

Later on, samples were taken to determine PC and APC content using the previously described method[28]. The following formulas were used to calculate PC and APC, respectively.

$$PC = (0.162 \times OD615 - 0.099 \times OD650 - 0.001 \times OD565) \times V / W \quad \ldots (3)$$

$$APC = (0.171 \times OD650 - 0.0006 \times OD562 - 0.004 \times OD615) \times V / W \quad \ldots (4)$$

Where, V represents volume and W represents dry weight in the above formula.

Determination of carotenoids

Entire carotenoids were taken out from the pellets, obtained from the samples afterward centrifugation. Taking out was completed with acetone (80 % v/v) in the dark at 4 °C for 24 h. Extracts were centrifuged for 20 min at 3,000 rpm, and absorption was calculated at 480 and 510 nm (total carotenoids). Pigments concentrations were calculated via published extinction coefficients and equations[29].

$$7.6(E_{480}\text{-}1.49E_{510}) \quad \ldots (5)$$

Where, E is the absorbance at 480 and 510 nm.

Statistical analysis

Data for pigments content were analyzed using a paired *t*-test of GraphPad Prism 6. Mean values as well as standard deviations were determined for each treatment samples. The significance of the mean values of the triplicates for each of the control and treatment samples was determined using a *t*-test. This test was also used for analyzing different pigment ratios.

Results

Chl-*a* content

The chl-*a* content of the *P. yezoensis* was remarkably affected by *L. paradoxa* (Fig. 1). In contrast to control samples (13.47±0.6138 µg g^{-1}), the chl-*a* content in treatment samples (10.83±0.6974 µg g^{-1}) was significantly decreased ($p < 0.05$).

Phycoerythrin (PE) content

The PE content was also influenced by the epiphytism of *L. paradoxa* on *P. yezoensis* (Table 1 & Fig. 2a). The PE content of the treatment samples (6.990±0.0234 mg g^{-1}) was significantly increased compared to the control samples which was 4.007±0.06227 mg g^{-1} ($P < 0.001$).

Phycocyanin (PC) and Allophycocyanin (APC) content

The results demonstrated the PC and APC contents were also dramatically influenced by the epiphytic diatom. The results showed that PC content of the treatment samples (5.314±0.236 mg g^{-1}) was significantly increased in comparison to the control samples (3.540±0.158 mg g^{-1}) ($P < 0.01$, Table 1 & Fig. 2b). Besides, the statistical analysis showed that APC content in the treatment samples

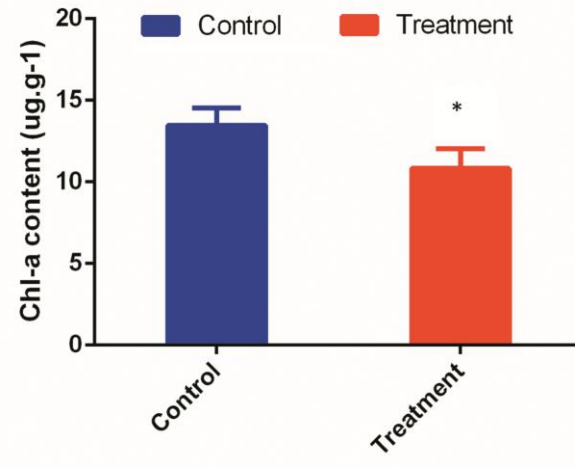

Fig. 1 — Chlorophyll-*a* content in the control and treatment samples. The indicated values are mean ± S.D of three replicates (*$p < 0.05$)

Table 1 — Comparison between the pigments content of different treatments of *P. yezoensis* represented by the results of *t*-test

Treatments	Parameter	*P*-values	Significance
Control vs treatment	Chl-*a*	0.006	Yes
	PE	0.010	Yes
	PC	0.030	Yes
	APC	0.001	Yes
	Carotenoid	0.0006	Yes

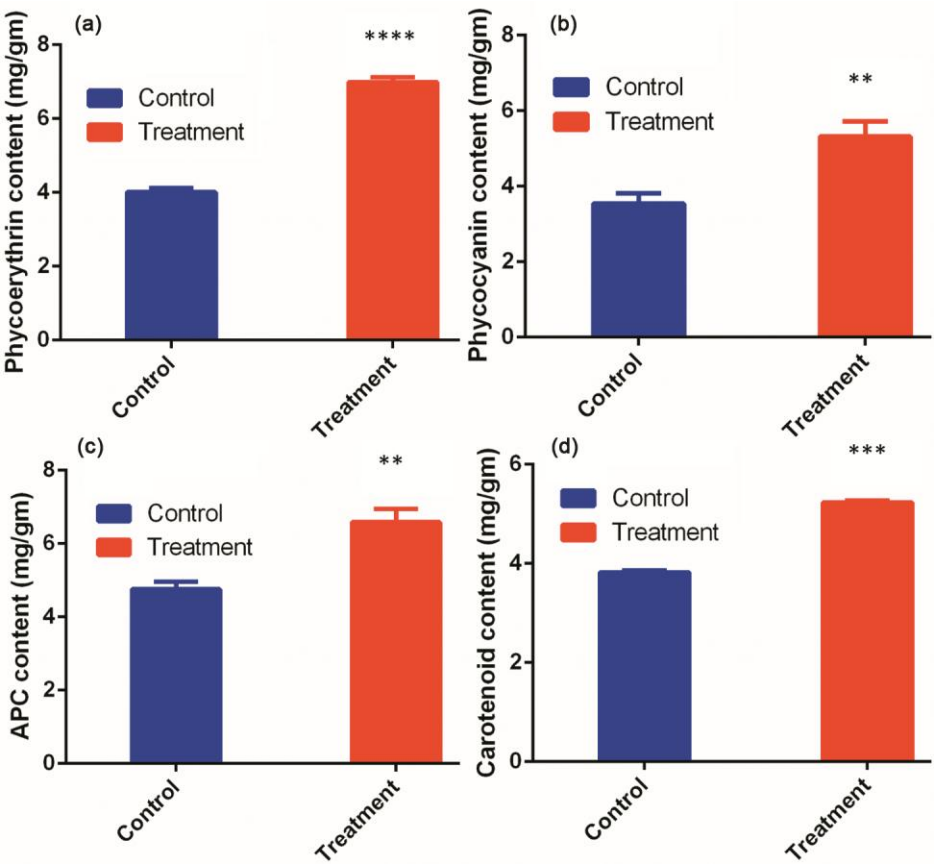

Fig. 2 — Effects of epiphytism on the pigments contents of *P. yezoensis*: (a) Phycoerythrin (PE) contents; (b) Phycocyanin (PC) contents; (c) Allophycocyanin (APC); and (d) Carotenoid (Car) contents in control and treatment samples. Indicated values were mean ±S.D of three replicates (**p < 0.01, ****p < 0.001, *p < 0.05, ***p < 0.005)

(6.583±0.213 mg g^{-1}) was significantly increased as compared to control samples (4.755±0.118 mg g^{-1}) (P < 0.01, Table 1 & Fig. 2c).

Carotenoids content

Similarly, the epiphytes also affected the carotenoids contents of *P. yezoensis*. The results showed that carotenoid content in treatment samples (5.256±0.024 mg g^{-1}) was significantly increased as compared to control samples (3.846±0.024 mg g^{-1}) (P < 0.005, Table 1 & Fig. 2d).

Pigments ratios

The ratios of different pigments to cholorophyll-*a*, such as APC/Chl-*a*, Car/Chl-*a*, PC/Chl-*a* and PE/Chl-*a* increased in the treatment samples as shown in Table 2. The ratio of these accessory pigments to Chl-*a* increased due to the light intensity decreased by epiphytism phenomenon.

The following results showed highly significant values between treatment and the control samples. The results demonstrated a clear increase in the ratios of the Car/Chl-*a*, PC/APC, PC/Car, PC/Chl-*a*, APC/Chl-*a* and PE/Chl-*a*, while a decrease in the ratios of APC/PE, Car/APC, Car/PE and PC/PE in the treatment samples were observed. The highest pigment ratio PE/Chl-*a* was recorded among all the pigment ratios in the treatment samples.

Table 2 — Pigments ratios in treatment and controlled samples and the results of *t*-test (*p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001)

Pigments	Control	Treatment	Significance (P < 0.05)
APC/Chl-*a*****	0.010	0.0134	0.0001
APC/PE*	1.186	0.940	0.0218
Car/APC*	0.803	0.794	0.0207
Car/PE**	0.953	0.748	0.0095
Car/Chl-*a*****	317.5	520	0.0001
PC/APC	0.744	0.807	0.0720 (Non-significant)
PC/Car**	0.926	1.015	0.0068
PC/Chl-*a****	295.25	531	0.0001
PC/PE**	0.883	0.760	0.013
PE/Chl-*a*****	396.0	1097	0.0001

Discussion

The present study indicated that epiphytic diatom *Licmophora paradoxa* may have adverse effects on the growth pigments of the host *P. yezoensis*. The growth of epiphytic organisms on the exterior of macro alga is abundant in marine environments, which is known as the main problem throughout the world in seaweed farming as they may reduce the production and results in financial loss[30-33].

Macroalgae are mutual hosts for epiphytic diatoms[34-36]. They can invade macroalgae and cause harmful effects. Most prominently, epiphytic organisms shade the light required for macro plants which cause a reduction in the photosynthetic efficacy which in-turn cause decrease in the production and reproduction[10-12]. The intervention in the photosynthesis can cause in the primary loss of submerged macrophytes[2,3,13]. The decrease of light intensity because of the epiphytic organism has been previously described in various studies[37-40]. Epiphytes may similarly assume such type of mechanisms to produce negative impacts on photosynthetic organelles thus reducing the chlorophyll-*a* (Chl-*a*) concentration of macrophytes[3,4,6]. This can mainly contribute to the interruption of light and nutrients by the diatoms affecting the growth of macroalgae[41]. As showed in present study, the influence of light availability for the host by the well-known and dominant diatom *Licmophora pardoxa*; the previous study also showed that the epiphytic diatom could cause negative impacts on incident light availability and photosynthetic pigments of *P. yezoensis*[7]. In previous study, it was reported that even low loads of epiphytes resulted in a distinct reduction of the light available for growth of the host plant[7,23]. Critchley *et al*.[31], reported that increased epiphytic burden on the leaves of native macrophytes are especially problematic as the microalgae stop incident light required for photosynthesis of macrophytes. The Present research showed that the diatom *L. paradoxa* covered the surface of *P. yezoensis* hindering the host from getting enough lights. So, this could be the basic reason of affecting the pigments content in the *P. yezoensis* due to epiphytism. Photosynthetic pigments like chl-*a* and Phycobiliprotien content in *P. yezoensis* were influenced significantly due to the attached diatoms on its surface. Phycobiliproteins which act as antenna pigments in Rhodophyta, allow them to do photosynthesis efficiently in water where blue light dominates[25].

The chlorophyll-*a* content significantly decreased in the treatment samples, showing negative impact of the diatom on the *P. yezoensis*. The present results of chl-*a* agree with the previous studies which stated that chl-*a* content could be decreased with high loads of epiphytic algae as compared to low loads of epiphytic algae on *Vallisneria natans*[6]. The previous studies also showed a clear reduction in the chl-*a* in *Gracilaria bursa-pastoris* as a result of low light[42], which supports the present study as the attachment of diatoms on *P. yezoensis* surface cause reduction of light intensity, resulting in decreased chl-*a* content.

The key photosynthetic accessory pigments are the phycobiliproteins of the red macroalgae. *P. yezoensis* includes PE, PC, and APC as main photosynthetic pigments. In this study, phycobiliproteins content was significantly increased in the treatment samples, which shows similar results to the work of Pang *et al*.[23] and Marinho-Soriano[43]. These results could be attributed to low light conditions because of the attachment of diatom cells on the surface of the *P. yezoensis.* Pang *et al*.[23] showed that PSII performance of *Kappaphycus alvarezii* was affected by the stress produced by *Neosiphonia savatieri* and hence the seaweed adapted itself to the reduced light state via increasing its Phycobiliproteins to capture extra light energy. Marinho-Soriano[42] reported an increase in Phycobiliprotein content in *Gracilaria bursa-pastoris* as a result of the low light intensity in the depth of sea[42]. The increase was because of the adaptation mechanism of the alga to low light intensity and its usage of the pigment contents as reserves for continuing the growth in stress condition. Thus the increase in the pigments could be to overcome the stress and to continue the growth.

Phycobiliproteins, which act as antenna pigments in rhodophyta, and enable this group to do photosynthesis effectively in deep water where blue light predominates[22]. In the present study, PE was remarkably increased in the treatment samples against the control ones, showing similar results to the previous studies[23,42], that the reduction of the light intensity with distance added to the rise in the pigment content of the algae. The opposite association amongst light intensity and PE content has been described; where the previous studies of Pang *et al*.[23] and Marinho-Soriano[42] indicated that the light intensity is an important aspect in the improved concentration of the pigment content with deepness in *G. bursa-pastoris.*

Phycobiliprotein have an internal core of APC, while phycocyanin PC is present as several intermediate packets. PC and APC also showed significant increase in the treatment samples against the control ones ($P < 0.05$), which is similar to the earlier results[43].

This happens due to little light conditions, a larger quantity of pigment is needed to improve the chance of photons being captured through the antenna molecules of the photosystems. As a result of the epiphytism, the epiphytic diatom *L. paradoxa* caused shading of the *P. yezoensis*, reducing the availability of light for the host. The present study showed that the host *P. yezoensis* adapted itself to reduce the consequences of the low light by increasing the phycobiliprotien showing similarity to the earlier reported study[21]. The previous study reported that phycobiliprotien, could contribute in a very effective energy transmission series in the reaction centers of PSII, and liable for around 50 % of light catching in the cyanobacteria and red algae[44].

Overall, the phycobiliprotein content has been increased in *P. yezoensis* in the treatment samples. Similar results have also been found for *Gracilaria lemaneiformis*[45] and for *Gracilaria* chilensis[46] that the increase in accessory pigment to overcome the decrease in light intensity concentration with the adaptation approaches used by the algae to use further light for photosynthesis and eventually to overcome the stress such as low incident light due to epiphytism phenomenon, to optimize its metabolic rate.

In the present study, pigment ratios of the control and treatment samples have been determined and indicated the effects of epiphytism on the pigments content of the hos, which showed influence on the pigment contents. The role of these ratios of different pigments to Chl-*a*, particularly (APC/Chl-*a*, Car/Chl-*a*, PC/Chl-*a* and PE/Chl-*a*), where the ratios of these accessory pigments to Chl-*a* increased significantly due to decrease in light intensity by epiphytism phenomenon against the normal condition, where these ratios should be lower than 1 and hence the Chl-*a* is higher than these accessory pigments. For instance the increase in carotenoid content and consequently Car/Chl-*a* under this epiphytic situation implied an enhancement of its assisted function in light-harvesting, photo protection and stress-tolerance mechanism.

On the other hand, the increase in carotenoid content in the treatment samples are supported by the previous studies[41], which observed that shading by diatoms can reduce the amount of light intensity which caused an increase in the total amount of carotenoid of the *Gracilaria chilensis*.

Conclusion

The present study proved the view that epiphytic diatom *Licmophora paradoxa* could seriously affect the pigments content of the host *P. yezoensis*. These results suggest that epiphytes may influence the pigments of macroalgae by decreasing the available light and decreasing Chl-*a* while increasing other pigments such as PE, PC, APC and carotenoids. It indicated that under lower light shaded by epiphytic diatoms, *P. yezoensis* could compensate the negative effect on photosynthetic reaction center pigments, chlorophylls by enhancing those antenna pigments to capture more light energy. Thus, the study contributes in understanding the relationship between macro and micro algae. The study also suggests further investigation on physiological and molecular level.

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Conflict of Interest

There is no conflict of interest among all the authors.

Author Contributions

TUK carried out all the experiments under the supervision of GD, SK carried out revision and submission and SC, AA & AK contributed in designing and analysis of the results and manuscript writing.

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