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Chlorophyll *a*: *b* Ratio Increases Under Low-light in 'Shade-tolerant' *Euglena gracilis*

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ABSTRACT. *Shade tolerance is a key adaptive strategy displayed by heliophytic photosynthetic organisms in response to limited light. Although generalized morphological and physiological traits associated with shade tolerance exist, the interest in shade tolerance has been expanding over the past few years due primarily to the controversies that have emerged on classical hypotheses of shade tolerance. In this paper the shade responses of unicellular excavate Euglena gracilis is discussed. Euglena was photoautotrophically grown under three different light intensities; 28, 84 and 210 µmol m^{-2} s^{-1}. Results revealed that E. gracilis is a shade tolerant species which exhibits some typical shade tolerant responses such as decrease in growth rate, light saturation point, light compensation point and dark respiration rate, and increased chlorophyll content. Most importantly, it is reported for the first time that the shade tolerance of this organism is also characterized by the increased chlorophyll a:b ratio, contradicting the generally accepted hypothesis of decreased chlorophyll a:b in shade tolerance response. The probable reasons for increased chlorophyll a:b ratio in E. gracilis under shade are also discussed.*

Key words*: Chlorophyll, Fluorescence, Photosynthesis, PSI, PSII.*

INTRODUCTION

Photosynthetic apparatus of organisms adapt to low light environments allowing coordinated allocation of resources not only to achieve and maintain optimal rates of photosynthesis, but also to function efficiently under limited light (Anderson *et al.*, 1995). Shade tolerance is a key adaptive strategy that some heliophytic photosynthetic organisms show in response to low light. From an ecological point of view, shade tolerance refers to the capacity of a given photosynthetic organism to tolerate low light levels (Valladares and Niinemets, 2008) and it is typically characterized by a set of morphological and physiological traits such as decrease in growth rate, light compensation point, dark respiration rate, net photosynthetic rate and chlorophyllv *a*:*b* ratio, and increase in quantum yield, chlorophyll content (both per area and per dry mass basis) and carbohydrate storage together with many other traits (Valladares and Niinemets, 2008).

Shade tolerance can be considered as a crucial life-history trait that plays a pivotal role in community dynamics of photosynthetic organisms. The success or failure in habitat selection is therefore governed by the extent to which the species can tolerate shade. Most importantly, the shade tolerance of species plays a central role in the functioning of CO_2-elevated future

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plant communities because elevated CO_2 greatly enhances the growth and photosynthesis of shade tolerant species (Naumburg and Ellsworth, 2000). This emerging understanding of the importance of shade tolerance in climate change together with the new findings regarding features involved in shade tolerance, which sometimes are controversial to the classical hypotheses, has triggered the expanding interest in shade tolerance in the recent years, although the topic of shade tolerance has a long history (Valladares and Niinemets, 2008)

Controversies exist over the mechanisms and traits conferring shade-tolerance (Kitajima, 1994; Valladares and Niinemets, 2008). Confounding ontogenetic effects have often been observed which alleviate the controversy. In the present work, it was intended to investigate the shade responses of unicellular excavate flagellate *Euglena gracilis*, a photosynthetic organism that does not possess ontogenetic effects. In addition to its significance as a source for production of antioxidants such as β-carotene, α-tocopherol and vitamin C (Takeyama *et al.*, 1997) and as a source of highly unsaturated fatty acids such as EPA and DHA (Hayashi *et al.*, 1993), *Euglena* has gained attention in the recent past as a source for production of bio-fuels in an economically effective and environmentally sustainable manner (Li *et al.*, 2008). Knowledge on shade responses of this excavate will surely benefit the commercial cultivation as well.

Euglena has long been considered as an extreme type of 'sun-alga' (Brody, 1968). Although Cook's (1963) comprehensive study dealt with growth responses as affected by light energy supply, little is known about how *Euglena* adapts to varying light intensities, low light levels in particular, with respect to physiological parameters and photosynthetic mechanism. The objective of the current study was to determine if *Euglena gracilis* is a shade tolerant species, and identify the traits that characterize the shade tolerance (or intolerance).

MATERIALS AND METHODS

Organism and culture conditions

Euglena gracilis Klebs strain Z provided by Dr. L. Edmunds of Stony Brook, NY, USA, was cultured photoautotrophically and axenically in modified Cramer-Meyer medium according to Bolige *et al.* (2005) at 25˚C as batch cultures. Cultures were continuously irradiated unilaterally by an array of day-white type fluorescent lamps (National FL20SS-N18, Tokyo, Japan); irradiances used were 28, 84 and 210 μmol $m^{-2}s^{-1}$. All three cultures were magnetically stirred and aerated throughout the experimental period.

Growth analysis

The cell population growth was monitored by counting the cell number progressively in each culture with an electronic particle counter (Coulter Electronics, Inc., Hialeah, FL, USA). For this purpose, a volume of 5 mL was drawn at each sampling point using a fraction collector (SF-2120, Advantec). Specific growth rate (SGR) was calculated according to the following equation; $SGR = [\ln (N/ N_o)] / t$; where N_o is the cell count of the previous sampling, N is the cell count of the current sampling and t is the time between two samplings in hours.

Chlorophyll analysis

Four millilitres of 100% acetone was placed into a known concentrated cell titer of 1 mL and homogenized at 1000 rpm for one min. The homogenate was centrifuged at 2500 rpm for 10 min. The supernatant was separated and the absorbance was read at 400-700 nm on

Schimadzu UV-260 spectrophotometer. It was recorded that chlorophyll *a* showed the maximum absorbance at 662 nm and chlorophyll *b* at 646 nm and the amounts of these pigments were calculated according to the simultaneous equations of Lichtenthaler and Welburn (1983) as follows:

$$\text{Chl } a = 11.75\ A_{662} - 2.350\ A_{646}$$
$$\text{Chl } b = 18.61\ A_{646} - 3.960\ A_{662}$$

Measurement of photosynthesis and respiration

Photosynthetic O_2 evolution was measured as a function of light intensity in each culture at exponential, transitional and stationary phases, using an oxygen electrode (Digital Oxygen System, model 10, Rank Brothers, Cambridge, England) and recorded by a chart type recorder (Unicoder U-228, Pantos, Nippon Denshi Kagaku, Japan). For all measurements, a concentrated cell density of ~2 - 2.5 × 10^5 cells/mL was used in the chamber. The chamber was irradiated using a xenon light source (LAX-102, Asahi Spectra USA Inc.) of varying intensities. A constant temperature circulator was used to maintain the chamber temperature at 25˚C. The O_2 consumption in the dark (dark respiration) was also recorded.

Measurement of chlorophyll fluorescence

Chlorophyll fluorescence was measured in cell suspensions (2 mL) using AquaPen AP-C 100 fluorometer (PSI, Czech Republic). For each measurement, a cell titer of ~ 8 × 10^4 cells/mL was used. The samples were dark-adapted for 10 min; the reaction centers (RCs) of photosystem II (PSII) were completely oxidized in the dark (Strasser *et al.*, 2004). Immediately after dark-adaptation, the cells were exposed to a saturating light pulse of 3000 μmol m^{-2} s^{-1}. The fluorescent transients were recorded in a time span from 10 μs to 1 s at 10 μs intervals. Each transient was analyzed according to OJIP-test (Strasser *et al.*, 2004) by using raw data; F_0 - the fluorescence intensity at 50 μs when all RCs are open (O-step), F_K - at 300 μs (K-step), F_J - at 2 ms (J-step), F_I - at 30 ms (I-step) and F_M - maximum fluorescence intensity assuming all the RCs are closed by the saturating light pulse (P-step). Using these values, two other basic parameters were also derived; $M_0 = 4\ (F_K - F_0)/(F_M - F_0)$, $V_J = (F_J - F_0)/(F_M - F_0)$. The following equations were used to explain the PSII behavior (Strasser *et a.*, 2004): Number of photons absorbed (ABS) per cross section (CS) ABS/CS ≈ approximately proportional to F_0 (Strasser *et al.*, 2004); effective antenna size of an active reaction center ABS/RC $=(M_0/V_J)/[1-(F_0/F_M)]$; density of RCs per cross section RC/CS = (ABS/CS)/(ABS/RC); maximum quantum yield of primary photochemistry $\varphi_{P0}=[1-(F_0/F_M)]$.

Statistical analysis

For each variable, four replicates (independent samples) were obtained from each growth phase (at the middle of the exponential and transitional phases and at early stationary phase) for all three light treatments. The results were subjected to analysis of variance and the means were compared by the Tuckey test at 5% probability. The statistical analysis was performed using the SAS software, version 8.02 (SAS Institute, Inc., Cary, NC).

RESULTS

The shading effect on physiology and photosynthetic pigment composition of *Euglena gracilis* grown photoautotrophically was examined by comparing not only the cultures grown under three different incident photon flux densities (PFDs) but also gradual decrease of actual irradiance occurring within each culture by mutual shading with increasing cell

titer.

Growth characteristics

Figure 1 shows the time course of cell number increase in photoautotrophic batch cultures of *Euglena* as affected by light intensity. The cell population growth under all light intensities displayed initial log-linear exponential growth phase followed by transitional phase and finally reaching the stationary phase.

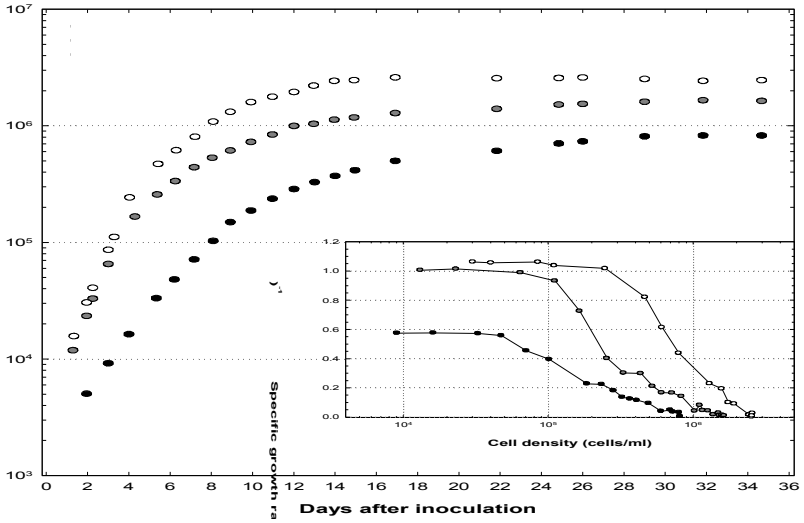

Fig. 1. Cell population growth of photoautotrophically grown *Euglena gracilis* period as affected by growth PFDs of cultures. Light intensities used: 28 (black circles), 84 (gray circles) and 210 (open circles) µmol $m^{-2}s^{-1}$. Inset shows the change in specific growth rate (see materials and methods) of each culture with increasing cell density.

The exponential phase, as evaluated by the phase of constant specific growth rates (Fig. 1-inset), ceased at ~2.5×10^5, ~1.4×10^5 and ~0.5×10^5 cells/mL PFDs of 210, 84 and 28 µmol m^{-2} s^{-1}, respectively. In respective cultures, the stationary phase was reached at cell densities of ~2.5×10^6, ~1.6×10^6 and ~0.85×10^6 cells/mL taking 14, 26 and 32 days after inoculating with the same amount of cells respectively; the higher the light intensity, the higher the stationary cell titers and shorter the time taken to reach the stationary phase. As shown in the inset of Fig.1 and in Table 1, lower light intensities (among cultures and within each culture after post-exponential phases) resulted in decreased growth rates.

Photosynthesis and respiration

The plots of the change of net photosynthetic oxygen evolution rate with PFD in exponential, transitional and stationary cultures of the three different light intensities were established (Fig. 2). During the exponential growth phase of each culture (Fig. 2a and Table 1), the values of dark-respiration rate, light-compensation and light-saturation points were significantly lower (P<0.05) in lower light cultures. These differences across cultures were however not maintained until stationary phases, because actual PFD became unparallel to incident PFD due largely to mutual shading; higher incident PFD supports higher cell titers with higher speeds, leading to heavier mutual shading. Thus, in the transitional growth phase (Fig. 2b), the lowest light-saturation point was achieved in *Euglena* cultured at the highest

incident PFD, whereas the values of both the dark-respiration rate and the light-compensation point still followed the same as in the exponential cultures; the lower in the lower incident PFD cultures. When they reach the stationary growth phases (Fig. 2c), all these variables became indistinguishable from each other; all the cultures may have encountered deep shade below a critical level, such that the actual PFD, although physically not the same, were essentially (or biologically) the same for the cells. In spite of these complexities, it is obvious that all the three variables, i.e. dark-respiration rate, light-compensation- and light-saturation points are lower in lower PFD cultures and decreased with increasing cell titers, thus decreasing actual PFD, within each culture (Table 1).

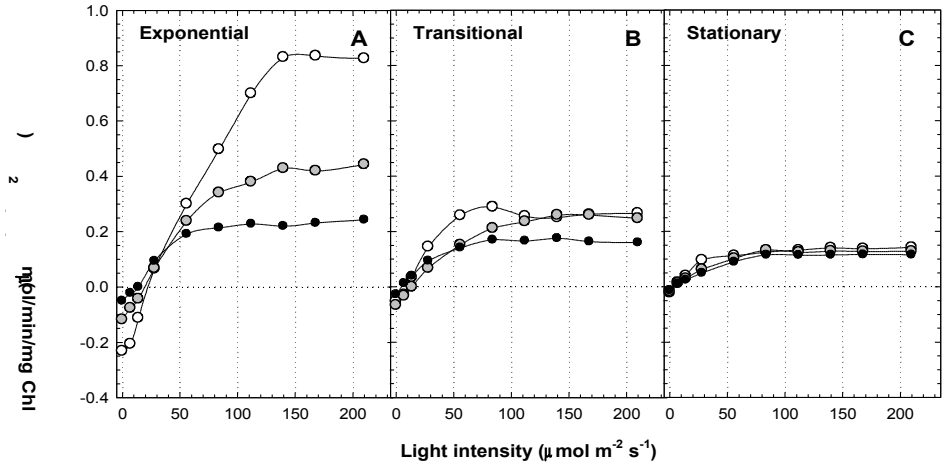

Fig. 2. Photosynthetic oxygen evolution of photoautotrophic cultures of *Euglena gracilis* as affected by incident PFD. Three light intensities have been used; 28 (black circles), 84 (gray circles) and 210 (open circles) µmol $m^{-2}s^{-1}$. (A) Exponential phase (B) transitional phase and (C) stationary phase.

Table 1. Light-shade responses of *Euglena gracilis* under three different light intensities in three growth phases.

Response	Growth stage	Light intensity		
		28 µmol.m^{-2}.s^{-1}	84 µmol.m^{-2}.s^{-1}	210 µmol.m^{-2}.s^{-1}
Growth rate	Exponential	0.576 ± 0.003^{cp}	1.008 ± 0.002^{bp}	1.056 ± 0.003^{ap}
(day-1)	Transitional	0.181 ± 0.003^{cq}	0.211 ± 0.002^{bq}	0.360 ± 0.003^{aq}
	Stationary	0.011 ± 0^{ar}	0.015 ± 0.001^{ar}	0.012 ± 0.001^{ar}
Dark respiration rate	Exponential	1.379 ± 0.017^{cp}	2.151 ± 0.006^{bp}	2.894 ± 0.020^{ap}
(nmol/min/10^6 cells)	Transitional	0.759 ± 0.007^{bq}	1.323 ± 0.041^{aq}	0.733 ± 0.023^{bq}
	Stationary	0.486 ± 0.014^{ar}	0.496 ± 0.008^{ar}	0.503 ± 0.015^{ar}
Light compensation point	Exponential	14 ± 0.58^{cp}	20 ± 1^{bp}	23.4 ± 0.3^{cp}
(µmol.m^{-2}.s^{-1})	Transitional	5 ± 0^{cq}	13.7 ± 0.3^{aq}	11 ± 0.6^{bq}
	Stationary	4 ± 0^{ar}	4 ± 0^{ar}	4.2 ± 0.2^{ar}
Light saturation point	Exponential	110 ± 1.2^{bp}	141.3 ± 2^{ap}	140 ± 1.2^{ap}
(µmol.m^{-2}.s^{-1})	Transitional	84 ± 0.3^{bq}	142 ± 2^{ap}	85 ± 0.6^{bq}
	Stationary	83 ± 1.7^{aq}	84 ± 0.9^{aq}	84 ± 2^{aq}
Photosynthetic capacity	Exponential	0.232 ± 0.006^{cp}	0.421 ± 0.009^{bp}	0.83 ± 0.009^{ap}
(µmol/min/mg Chl)	Transitional	0.17 ± 0.002^{cq}	0.26 ± 0.006^{bq}	0.28 ± 0.003^{aq}
	Stationary	0.115 ± 0.006^{br}	0.128 ± 0.002^{ar}	0.14 ± 0.01^{ar}
Quantum yield	Exponential	0.61 ± 0.02^{aq}	0.54 ± 0.02^{bq}	0.45 ± 0.01^{cr}
	Transitional	0.65 ± 0^{ap}	0.62 ± 0.02^{bp}	0.57 ± 0^{cq}

	Stationary	0.65 ± 0.01^{ap}	0.62 ± 0.02^{bp}	0.62 ± 0.01^{bp}
Total chlorophyll content	Exponential	27.5 ± 1.3^{ar}	18.4 ± 0.7^{br}	12.7 ± 0.3^{cr}
(pg/cell)	Transitional	29.2 ± 0.3^{aq}	22.6 ± 0.8^{bq}	14.4 ± 0.8^{cq}
	Stationary	34.9 ± 1.1^{ap}	30.3 ± 0.3^{bp}	23.2 ± 1.1^{cp}
Chlorophyll a	Exponential	23.4 ± 1.3^{ar}	15.1 ± 0.7^{br}	10.2 ± 0.3^{cr}
(pg/cell)	Transitional	25.1 ± 0.3^{aq}	19.2 ± 0.9^{bq}	11.9 ± 0.7^{cq}
	Stationary	30.9 ± 1.1^{ap}	26.5 ± 0.3^{bp}	20.1 ± 1^{cp}
Chlorophyll b	Exponential	4.1 ± 0.1^{ap}	3.3 ± 0.1^{bq}	2.5 ± 0^{cq}
(pg/cell)	Transitional	4.1 ± 0.1^{ap}	3.4 ± 0.1^{bq}	2.5 ± 0.1^{cq}
	Stationary	4 ± 0.1^{ap}	3.9 ± 0^{ap}	3 ± 0.1^{bp}
Chlorophyll a:b ratio	Exponential	5.7 ± 0.4^{aq}	4.6 ± 0.2^{br}	4.1 ± 0.1^{cr}
	Transitional	6.1 ± 0.2^{aq}	5.7 ± 0.3^{aq}	4.9 ± 0.2^{bq}
	Stationary	7.7 ± 0.4^{ap}	6.8 ± 0^{bp}	6.6 ± 0.2^{bp}
ABS/CS	Exponential	211 ± 8^{ap}	229 ± 8^{ap}	225 ± 7^{ap}
(a.u.)	Transitional	225 ± 8^{ap}	219 ± 9^{ap}	194 ± 14^{aq}
	Stationary	221 ± 8^{ap}	211 ± 3^{ap}	198 ± 7^{aq}
ABS/RC	Exponential	2.1 ± 0.0^{cp}	2.9 ± 0.1^{bp}	4.1 ± 0.0^{ap}
(a.u.)	Transitional	1.4 ± 0.1^{cq}	1.7 ± 0.1^{bq}	2.1 ± 0.1^{aq}
	Stationary	1.3 ± 0.0^{cq}	1.4 ± 0.0^{br}	1.51 ± 0.1^{ar}
RC/CS	Exponential	102 ± 6^{aq}	80 ± 1^{br}	54 ± 1^{cr}
(a.u.)	Transitional	158 ± 11^{ap}	132 ± 1^{bq}	94 ± 5^{cq}
	Stationary	168 ± 4^{ap}	148 ± 4^{bp}	129 ± 2^{cp}

Mean values (n=4) ± SEM are shown. Within each variable, mean values followed by different letters (a-c) in rows, and mean values followed by different letters (p-r) in columns are significantly different (P<0.05); Tuckey test. ABS/CS: PSII chlorophyll pool size; ABS/RC: antenna size: RC/CS: density of reaction centers (see Materials and Methods for details).

The maximum quantum yield of primary photochemistry (Φ_{Po}) that measures the efficiency of PSII photochemistry was determined using OJIP fluorescence transients according to Strasser *et al.* (2004). Apparently, during the exponential growth phase, i.e. at the beginning of the culture, Φ_{Po} was the highest in the culture that received 28 µmol m^{-2} s^{-1} and decreased with increasing light intensity among cultures (Fig. 3 and Table 1). With increasing cell titer, thus reducing the incident PFD, the quantum yield increased slowly but steadily in 28 µmol $m^{-2}s^{-1}$, while in other two cultures Φ_{Po} remained constant during the exponential growth phase and a marked increase in Φ_{Po} was observed during the transitional phase.

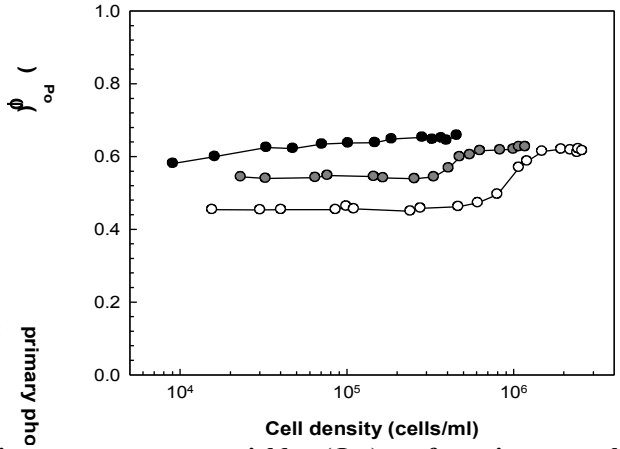

Fig. 3. Maximum quantum yield (Φ_{Po}) of primary photochemistry of photoautotrophically grown *Euglena gracilis* as affected by culture PFD.

Light intensities used were 28 (black circles), 84 (gray circles) and 210 (open circles) µmol $m^{-2}s^{-1}$

Chlorophyll pool

As shown in Fig. 4a, the total Chl (Chl *a*+*b*) not only exhibited higher cellular contents in cultures at low light intensities but also increased in their cellular contents with increasing cell titer leading to mutual shading, particularly after the transitional phase. Among the three PFD levels investigated in the present study, the proportion of total Chl relative to the dry mass (Chl/DM) was the highest in the 28 µmol $m^{-2}s^{-1}$ culture (Fig. 4b). Within each culture, the Chl/DM ratio was constant until the end of the exponential growth phase and thereafter it increased steadily, resulting in 2.75-, 4.2- and 4.7-fold increases by the stationary phase in 28, 84 and 210 µmol m^{-2} s^{-1} cultures, respectively.

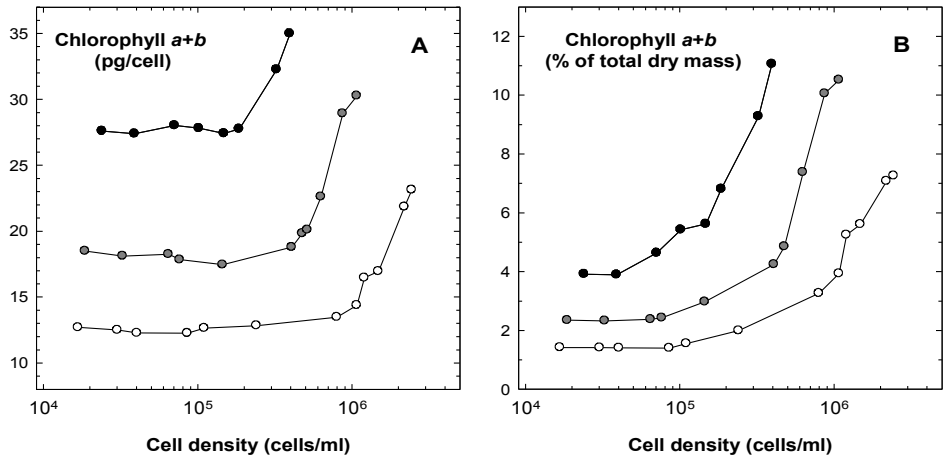

Fig. 4. Total chlorophyll content (Chl *a* + *b*) of photoautotrophically grown *Euglena gracilis* as affected by varying incident PFDs. A) as cellular basis (pg/cell). B) as percentage of DM (Chl/DM). Light intensities used were 28 (black circles), 84 (gray circles) and 210 (open circles) µmol $m^{-2}s^{-1}$.

Figure 5 shows the cellular chlorophyll *a* and *b* contents, and the Chl *a*:*b* ratio. Chl *a* showed a similar behavior (Fig. 5a and Table 1) as was observed in total chlorophyll content. During the exponential growth phase Chl *b* content was significantly different (P<0.05) among cultures; lower the PFD, higher the Chl *b* in cells (Fig. 5b). In both 84 and 210 µmol $m^{-2}s^{-1}$ cultures, the Chl *b* content sharply increased by ~20-25% during the mid-transitional phase, but in 28 µmol m^{-2} s^{-1} culture, it remained unchanged throughout the experimental period. Compared to Chl *a*, Chl *b* content was significantly lower (P<0.05) in cells under all three light intensities at any cell density (Table 1). As shown in the Fig. 5c, Chl *a*:*b* ratio increased with decreasing light intensity, both among cultures and within each culture with increasing cell titer. As the Chl *b* content was remarkably low compared to Chl *a* (Fig. 5a and b), the Chl *a*:*b* ratio appears to be determined solely by the amount of Chl *a* in *Euglena*. The maximum percentage of Chl *b* present in the total Chl pool in *Euglena* under current growth conditions was found to be ~19% and that was observed in exponentially growing cells cultured at 210 µmol m^{-2} s^{-1} PFD (Fig. 6). With increasing shade, not only among cultures, but also within each culture with increasing cell density, the percentage Chl *b* content in the total Chl pool decreased markedly indicating a greater increase in Chl *a* relative to the increase in Chl *b*.

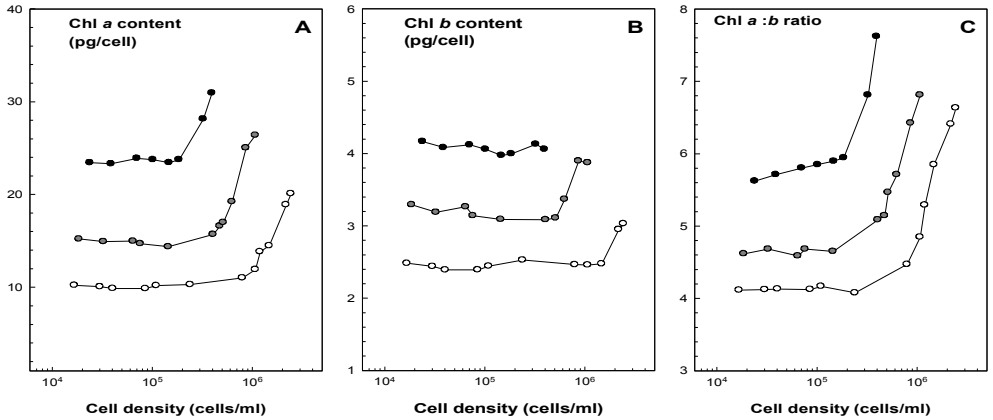

Fig. 5. Chlorophyll *a*, *b* and *a*:*b* ratio of *Euglena gracilis* as affected by incident PFD. (A) Chl *a*. (B) Chl *b*. (C) Chl *a*:*b* ratio. Light intensities used were 28 (black circles), 84 (gray circles) and 210 (open circles) µmol $m^{-2}s^{-1}$.

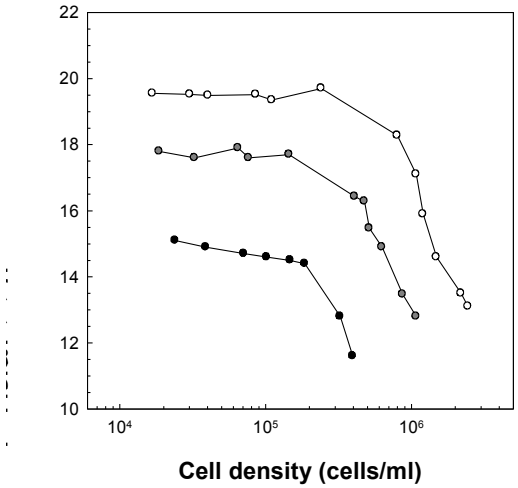

Fig. 6. Chlorophyll *b* content as a percentage of total chlorophyll pool in *Euglena gracilis* in response to varying growth PFDs. Light intensities used were, 28 (black circles), 84 (gray circles) and 210 (open circles) µmol m^{-2} s^{-1}.

OJIP analysis of PSII units

Chlorophyll *a* fluorescence transients were analyzed using the JIP-test according to Strasser *et al*. (2004) to determine the PSII structure and the composition. The number of photons absorbed by the antenna molecules of PSII reactions centers (RCs) over the excited cross section of the sample is represented by the ABS/CS_0 and this parameter is proportional to the Chl content (PSII) of the sample (Kruger *et al.,* 1997).

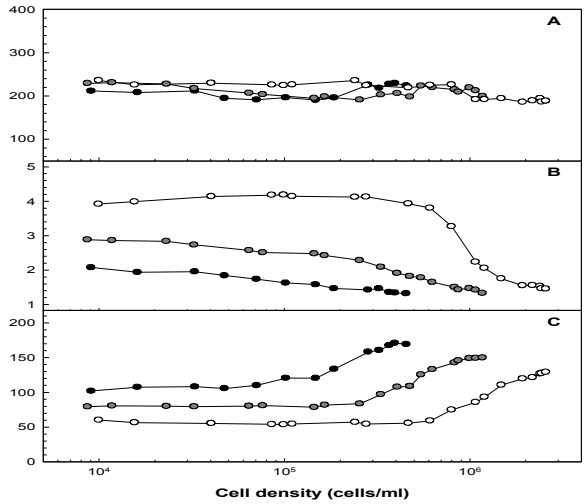

Fig. 7. PS II structure and composition related JIP-test parameters. (A) PS II chlorophyll pool size (ABS/CS), (B) effective antenna size of an active reaction center (ABS/RC), (C) reaction center density (RC/CS). Light intensities used were, 28 (black circles), 84 (gray circles) and 210 (open circles) µmol $m^{-2}s^{-1}$.

As shown in Fig. 7a, ABS/CS_0 did not change drastically either among cultures or within each culture with increasing cell density, suggesting relatively unchanged Chl content in PSII. However, spectrophotometric analysis of extracted Chl showed a marked increase in Chl content (Fig. 4a) in low-light cultures. Thus, it is likely that this increase in total Chl is a result the increase in PSI units (either size or number). The effective antenna size of an active RC of PSII (ABS/RC) was the highest in the cells grown at a PFD of 210 µmol m^{-2} s^{-1} (Fig. 7b) during the exponential growth phase, and after mid-transitional phase of the same culture, it decreased gradually. In the other two cultures, a gradual decrease in the antenna size with increasing cell titer was observed. The amount of active PSII reaction centers per excited cross section (RC/CS) was higher in cells grown under 28 µmol m^{-2} s^{-1} compared to other two cultures (Fig. 7c). In all the three cultures, the RC/CS remained constant until the early transitional phase and thereafter increased gradually.

DISCUSSION

Growth and shade tolerance

When the growth and shade tolerance of a species are to be correlated, it should be noted that the shade tolerant species are those facultatively adapted to shade and are different from obligate shade species that grow and reproduce under shade. Poorter (1999) suggested that higher shade tolerance of species is associated with lower potential growth under shade where 15 rain forest tree species have been used to demonstrate that species having low light compensation point (LCP) are characterized by low relative growth rate (RGR). Moreover, it has been shown that shade tolerant species may survive in the shaded habitats for years without a considerable growth (Turner, 1990). This is in accordance with the notion that the shade tolerance of a species is not related to growth but to persistence or survival in shade (Poorter, 1999). Agreeing to all the above, *Euglena* in this study showed a reduced growth

rate at lower light intensities and almost ceased the cell population growth (SGR≈0) at higher cell titers within each culture, i.e. at remarkably lower light levels within cultures. After reaching the stationary phase, the cell titer remained constant at least for about 3-5 weeks (data not shown) after which, counting the cell number became practically difficult as cell aggregation occurred. These results provide the first evidence for the shade tolerance of *Euglena*.

Photosynthesis, respiration and shade tolerance

Among other established characteristics of shade-tolerance of land plants are the decreased dark respiration, light compensation point and light saturation point (Valladares and Niinemets, 2008). Not only because these features are always seen in shade-tolerant but never in shade-intolerant land plants, but also because they clearly play pivotally functional roles in shade tolerance, they should represent the most fundamental criteria for shade tolerance.

Givnish (1988), in his 'carbon gain hypothesis', explains the shade tolerance as the maximization of light harvesting and efficient use of captured light in photosynthesis with decreased respiration costs for maintenance. According to this hypothesis, any trait that enhances the light use efficiency and hence the carbon gain, would increase the shade tolerance of a species. This study demonstrated that *Euglena* maximizes light capturing by increasing chlorophylls under low light (Fig. 4a). Moreover, the reduced light compensation point, which is a simple measure of shade tolerance and low dark respiration rates (Fig. 2) under low light have assisted *Euglena* to be efficient by reducing the losses.

These shade-tolerant features were also seen in *Euglena* when the plots of net photosynthesis rate vs. PFD in exponential cultures of the dimmest to the brightest-lit were compared (Fig. 2a). All the values of dark-respiration rate, light-compensation- and light-saturation points were lower in lower light cultures. It is therefore clear that *Euglena gracilis* is a shade-tolerant species.

Photosynthetic pigments

It has been widely accepted that photosynthetic pigments, mostly chlorophyll (*a* and *b*) tend to increase with decreasing irradiance to facilitate increased light harvesting in shade tolerant species (Givnish, 1988). In the present study, it was observed that the chlorophyll content increases in low light cultures and even within each culture with decreasing light availability as cell density increases. High chlorophyll contents under low light situations found in *Euglena* can therefore be considered as a first line of adaptive response to reduced light.

Apart from the total chlorophylls, the ratio of chlorophyll *a* to *b* (Chl *a*: *b*) has been a key parameter to judge the shade tolerance of a particular species (Givnish, 1988), in that, shade-tolerant species display a lower ratio under shade compared to their counterparts grown under high light environments. It has been shown that shade tolerant species produce a higher proportion of chlorophyll *b* relative to chlorophyll *a*, which leads to a lower Chl *a*: *b* ratio, to enhance the efficiency of blue light absorption in low light environments (Yamazaki *et al.*, 2005). *Euglena* in the present study responded in the opposite direction: higher Chl *a*: *b* values were obtained under lower light intensities (Fig. 5c). This finding challenges the validity of using low Chl *a*: *b* ratio as an indicator of shade tolerance of species in general. Increased Chl *a*: *b* ratio in response to shade in the present study is the first evidence of this

nature. Plentiful studies report decreased Chl *a*: *b* ratio in response to shade (e.g. Kotzabasis *et al.*, 1999), and a few studies report an unchanged Chl *a*: *b* ratios in the light gradient continuum (e.g. Murchie and Horton, 1998). In support of the latter, on the other hand, Johnson *et al.* (1993) and Murchie and Horton (1998) showed only a weak association between Chl *a*: *b* ratio and shade tolerance. To add to the controversy of issue on Chl *a*: *b* ratio and shade tolerance, this study revealed that Chl *a*: *b* ratio increased under low light in response to shade in shade-tolerant *Euglena*. Therefore, it is proposed that the changes in Chl *a*: *b* ratios depending on the light environment might be a characteristic of species themselves.

Another striking feature related to Chl *a*: *b* ratio in *Euglena* was the presence of remarkably higher values. In the present study, Chl *a*: *b* ratios in the range of 4.1-7.6 were obtained (Fig. 6c). These values are in accordance with the previously reported values for *Euglena* (Cook, 1963), however, higher compared with the values reported by Brandt and Wilhelm (1990). Vascular land plants (Johnson *et al.*, 1993) and algae (Humbeck *et al*., 1988) usually have Chl *a*: *b* ratios in the range of ~1.5 - 4.2 irrespective of the light environments within which they are inhabiting. Seldom higher ratios can be found in literature; Kotazabasis *et al.* (1999) reported a Chl *a*: *b* ratio of ~5.6 in *Scenedesmus*, a unicellular green alga, and Falkowsky and Owens (1980) reported values of 5.6 and 6.7 for *Dunaliella* and *Chlorella vulgaris,* respectively, all values are for high-light grown cells. *Euglena* has been considered as an extreme type of 'sun-alga' because of their higher chlorophyll *a*: *b* ratios (Brody, 1968). However, according to our results, *Euglena gracilis* is no more an extreme type of 'sun-alga', instead a well shade tolerant species.

Photosynthetic units of *Euglena* in response to low light

Land plants usually add more antenna chlorophyll to PS II (increased antenna size) or increase the PS II reaction centers (RCs) relative to PS I RCs in response to shade (Ehleringer, 2006) to enhance light capture and energy transfer. However, Chow *et al.* (1988) demonstrated a decreased PS II RCs (or increased PS I RCs) under shade. Falkowski and Owens (1980) identified two contrasting strategies in low-light adaptations in marine phytoplanktons; increase in the size of PS I units (comprising RCs and antenna) in *Skeletonoma* sp., a diatom, and increase in number of PS I units in *Dunaliella*, a chlorophyte. Green algae *Scenedesmus* and *Chlorella* responded to low light in such a way that number of RCs of both PS II and PS I increased while the antenna size unaltered (Humbeck *et al.*, 1988).

Based on these results, it can be suggested that *Euglena* increases the PS II reaction centers (Fig. 7C) and decreases the antenna size of PS II (Fig. 7B) under low light conditions. As there was no drastic change in ABS/CS among cultures (Fig. 7A), this indicates that the amount of chlorophyll present in PS II does not drastically vary depending on the light availability. In the spectrophotometric analysis, however, we observed a marked increase in total chlorophyll in cells in response to decreased light (Fig. 4A). This suggests, *Euglena* ither increase the size or increase the number of PS I units under low light conditions giving rise an increased total chlorophylls. Increase in PS I units (size or number) explains the increased Chl *a*: *b* ratio under low light, as PS I often does not contain Chl *b* (Hirashima *et al.*, 2006). On the other hand, increased number of PS II RCs also supports the higher Chl *a*: *b* ratio under low light, as ~85% of Chl *b* in *Euglena* is confined to antenna (Cunningham and Sciff, 1986). As a whole, the shade tolerance response of *Euglena* in relation to photosynthetic units is characterized by increase in number of RCs in PS II, decrease in

antenna size of PS II, and increase in either number of PS I RCs or the antenna size of PS I (Fig. 8).

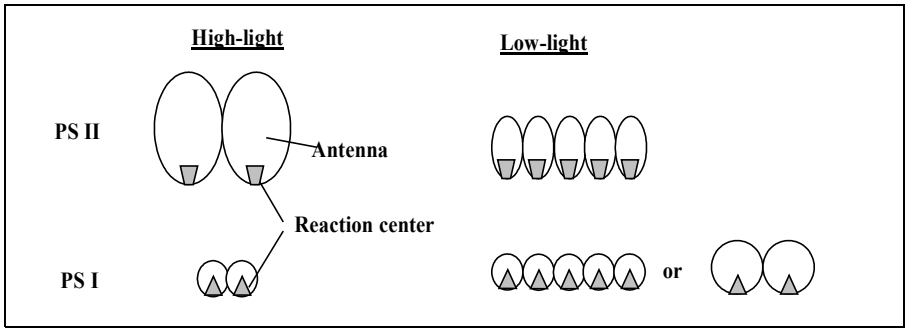

Fig. 8. Schematic representation of the organization of the photosynthetic apparatus in *Euglena gracilis* adapted to high and low light conditions.

CONCLUSION

It is concluded that *E. gracilis* expresses some typical shade-tolerant responses that have been generally adopted as the criteria for shade tolerance of land plants; decreased growth rate, light saturation point, light compensation point and dark respiration rate together with increased chlorophyll content. We have shown that, although *E. gracilis* to be a shade-tolerant species, its Chl a:b ratio increases with decreasing light intensities. Therefore, it is suggested that decreased Chl a:b ratio may not be taken as a generalized shade tolerant response for all photosynthetic organisms.

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