Differential Changes in the Photosynthetic Pigments and Polyamine Content during Photoadaptation and Photoinhibition in the Unicellular Green Alga *Scenedesmus obliquus*

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In the unicellular green alga *Scenedesmus obliquus* the level of photoinhibition and the recovery of the cells after reversal to the initial light conditions in relation to the pre-photoadaptation of the culture to low, medium and high light intensity was determined. The changes in the photosynthetic pigment content and in the intracellular polyamine concentration allowed to distinguish between photoadaptation and photoinhibition. In particular, the level of chlorophylls, xanthophylls and carotenoids decreased inversely proportional to the light intensity applied during photoadaptation, whereas their concentrations remained constant during photoinhibition. The violaxanthin/zeaxanthin and the loroxanthin/lutein cycle work only under photoinhibitory conditions, but not under photoadaptive premises. Changes in the level of these carotenoids in relation to the changes in the photosynthetic apparatus during photoadaptation are discussed. In addition, it was found that the intracellular polyamine level increased only under stress conditions, i. e. during photoinhibition, and decreased during recovery of the cells after reversal to the initial light conditions. The increase of the putrescine level during photoinhibition is inversely proportional to the light intensity used for pre-adaptation. This rise of the polyamine level in the cells photoadapted to high light conditions is an additional indication for the finding that photoadaptation and photoinhibition are different phenomena which are clearly distinguishable from each other. Finally, the changes of the chlorophyll, violaxanthin, zeaxanthin, loroxanthin, lutein and polyamine levels under photoadaptation in high light intensity (50 Wm^{-2}) in relation to the range of photoadaptation in *Scenedesmus obliquus* are discussed.

Introduction

It is a well documented fact that the photosynthetic apparatus contains not only chlorophyll *a* (Chl *a*) and *b* (Chl *b*), but also a great variety of carotenoids including α- and β-carotene, lutein, violaxanthin, zeaxanthin, loroxanthin and neoxanthin (Senger *et al.*, 1993). These carotenoids are distributed among the various Chl-protein complexes in a diverse pattern with β-carotene predominating in the reaction centers of the photosystems I (PS I) and II (PS II), the xanthophylls predominating in the proximal and distal antennae (Siefermann-Harms, 1985). It is known that higher plants (Anderson, 1986) and green algae (Fleischhacker and Senger, 1978) adapt to different light intensities accompanied by changes in the photosynthetic capacity and Chl content (photoadaptation). High light intensities lead to an inactivation of the photosynthetic electron transport and subsequent oxidative damage of the reaction center of PS II, in particular of the D1 protein (Aro *et al.*, 1993). In order to minimize photoinhibitory damage nature has evolved several mechanisms that serve to protect PS II under potentially damaging light conditions. One of the most important mechanisms is the capability of plants to dissipate excess excitation energy as heat. This phenomenon is related to the creation of a proton gradient across the thylakoid membrane and probably also to the formation of zeaxanthin (Demmig-Adams,

Abbreviations: Put, putrescine; Spd, spermidine; NorSpd, norspermidine; F_v, variable fluorescence; F_{max}, maximum fluorescence; Chl, chlorophyll; lut, lutein; loro, loroxanthin; vio, violaxanthin; zea, zeaxanthin.

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1990). The delimination of photoinhibition and photoadaptation is subject of the present work and is measured as the level of photoinhibition in dependence of pre-photoadaptation in low, medium and high light intensity which again correlates to the amount of photosynthetic pigments. Parallel to the pigment measurements changes in the intracellular level of polyamines will be quantified, as it is known that the amount of polyamines is an indicator for environmental and stress conditions. The variation of the level of polyamines under different growth conditions thus suggests an adaptive and protective role for these compounds (Smith, 1985). Furthermore, all photosynthetic pigment-protein subcomplexes, even the reaction center of PS II, contain polyamines (Kotzabasis *et al.*, 1993a). In addition, polyamines regulate Chl biosynthesis (Beigbeder and Kotzabasis, 1994), stabilize the thylakoid membranes, retard protein degradation and inhibit Chl decomposition (Besford *et al.*, 1991; 1993).

Materials and Methods

Organism, growth and illumination

Cultures of the unicellular green alga *Scenedesmus obliquus*, wild type, strain D3 (Gaffron, 1939), were grown autotrophically in liquid culture (Bishop and Senger, 1971) in a temperature-controlled water bath (30 °C) and illuminated by a set of white fluorescent lamps (L-40W, Osram, München, FRG). The cultures were prepared by inoculating fresh medium with a stock culture (2% (v/v) inoculum) and then continuously percolated with air enriched with 3% carbon dioxide.

For the photoadaptation experiments cultures were grown for 24 h in low (5 Wm^{-2}), medium (20 Wm^{-2}) and high (50 Wm^{-2}) light intensity. Prior to the photoinhibition experiments the cultures were also adapted to the above light intensities, divided into 10 ml portions of a cell density of 3.2 µl $PCVml^{-1}$ and then irradiated with light of an intensity of 1000 W m^{-2} for 45 min which yields a maximum of photoinhibition without causing photodestruction. This high intensity was achieved with a 250 W slide projector (Prado Universal, Leitz, Wetzlar, FRG) equipped with 250 mm Hektor lenses (Leitz). For recovery, cultures were transferred from the photoinhibitory to the initial light conditions for 90 min.

Polyamine analysis and estimation

The cells were harvested by centrifugation of the suspension at 3000×*g* for 10 min, the pellets suspended in 1 N NaOH in a proportion of 32 µl PCV per ml NaOH and then hydrolyzed according to the procedure of Tiburcio *et al.* (1985). 0.2 ml of the hydrolysate were mixed with 36% HCl in a proportion of 1:1 (v/v), transferred into ampoules, flame sealed and then hydrolyzed at 110 °C for 18 h. The hydrolysis products were centrifuged at 3000×*g* for 10 min to remove carbonized material and evaporated at 70–80 °C. The dried samples were redissolved in 0.2 ml of 5% (v/v) perchloric acid. To identify and estimate the polyamines, the samples were derivatized by benzoylation according to the modified method of Flores and Galston (1982). For that purpose 1 ml 2 N NaOH and 10 µl benzoylchloride were added to 0.2 ml of the polyamine containing hydrolysate and vortexed for 30 s. After a 20 min incubation at room temperature, 2 ml of saturated NaCl were added to stop the reaction. The benzoylpolyamines were extracted three times into 2–3 ml diethylether, all ether phases collected and evaporated to dryness. The remaining benzoylpolyamines were redissolved in 0.2 ml of 63% (v/v) methanol and 20 µl portions of this solution injected to HPLC for analysis of the polyamines according to the method of Kotzabasis *et al.* (1993b). The analyses were performed with a Hewlett-Packard 1090 HPLC equiped with a DPU multichannel integrator, a diode array detector (Hewlett Packard) and a narrow bore column (C_{18}, 2.1x200 mm, 5 µm particle size Hypersil; Hewlett Packard). To estimate directly the amount of each polyamine the method of Kotzabasis *et al.* (1993b) was applied.

Pigment extraction and quantitation

Total cell pigments were extracted from a standard volume of 32 µl PCV of algal cells by boiling for 1 min in methanol, centrifugation of the extract at 1400×*g* for 5 min and re-extraction of the pellet with hot methanol until it was colourless. The combined extracts were evaporated to dryness and redissolved in 3 ml of acetone. The Chl concentrations of the extracts were determined spectrophotometrically according to the method of Brouers and Michael-Wolwertz (1983). The concentrations of the individual carotenoids were de-

termined directly from the HPLC elution profiles. Calibration was performed with standard carotenoid samples (Humbeck *et al.*, 1988).

Pigment analysis by high performance liquid chromatography

Analysis of the pigment extracts obtained as outlined above was conducted with a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of two LC-6A solvent pumps and a SPD-6AV UV/VIS-spectrophotometric detector. 20 µl aliquots of the total cell extracts in aceton were administered to a 5 µm reversed phase main column (SS 200/6/4 Nucleosil $5C_{18}$, Macherey-Nagel, Düren, Germany) additionally equipped with a Lichrosorb RP18 (Merck, Darmstadt, Germany) guard column. The solvent flow rate was maintained at 1.0 ml/min. The solvent system consisted of initially 85% solvent A (acetonitile : methanol, 75:25) and 15% solvent B (double distilled water) which in the first 15 min was continuously increased to 92.5% solvent A and 7.5% solvent B and then to 100% solvent A within the next 25 min where it was maintained for an additional 20 min. The column was subsequently returned to the initial solvent composition of 85% solvent A and 15% solvent B within the next 11 min prior to the injection of a new sample (Humbeck *et al.*, 1988). Detection wavelenght was 445 nm.

Measurements of fluorescence induction

Fluorescence induction curves for the calculation of the ratio F_v/F_{max} after photoadaptation, photoinhibition and reversal to the initial light conditions were recorded with a custom-made PAM-fluorospectrophotometer, according to the method of Strasser and Strasser (1996).

Polarography

Rates of oxygen evolution at low, medium and high light intensities were determined polarographically at 30 °C with a micro Clark-electrode system (Gilson Medical Electronics Middleton, WI, USA). The light intensity was varied by inserting calibrated neutral density filters into the light beam. The cell density had been adjusted to 10 µl PCV/ml.

Determination of the packed cell volume

The packed cell volume (PCV) of a cell suspension was determined by centrifugation at $1400 \times g$ for 5 min using haematocrit tubes (Senger, 1970).

Results and Discussion

The aim of the present work was to establish criteria to distinguish between the reaction of the photosynthetic apparatus upon photoadaptation and photoinhibition. Additionally, the influence of the degree of photoadaptation on the mechanism of photoinhibition was investigated.

To elucidate the differentiation of cells during photoinhibition cultures pre-adapted to 5, 20 and 50 Wm^{-2} were exposed for 45 min to a light intensity of 1000 Wm^{-2} to cause maximum photoinhibition, but no photodestruction. Subsequently, the algae were transferred for 90 min back to the initial light conditions. Despite of the relatively short period of photoadaptation (24 h) to the different light intensities all cultures showed a similar behavior exhibiting a photosynthetic activity proportional to the applied light intensity (Fig. 1). During photoinhibition the ratio of F_v/F_{max} showed a similar fall for all three cultures which ranged between approximately 87 and 85% of the initial values. Cells adapted to 20 and 50 Wm^{-2} showed a recovery of up to 98–100% of the initial F_v/F_{max}-values. For the culture pre-adapted to low light in-

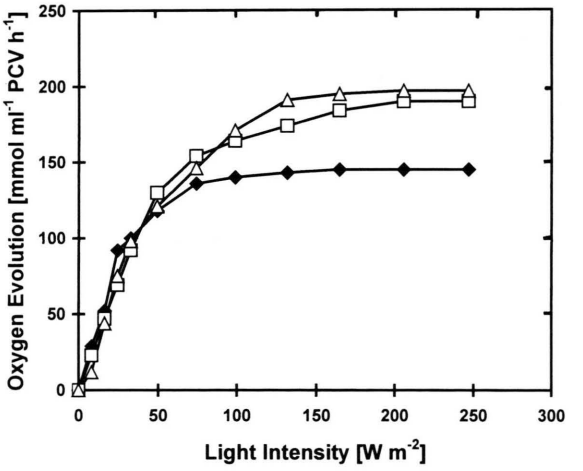

Fig. 1. Photosynthesis rates of cultures of *Scenedesmus obliquus* adapted in low (5 W m^{-2}, closed rhombes), medium (20 W m^{-2}, open squares) and high (50 W m^{-2}, triangels) light intensity for 24h.

tensity, the F_v/F_{max}-ratio after recovery did not overcome 94.5% of the initial value (data not shown). The ratios of F_v/F_{max} showed under these conditions very small differences. This may possibly be explained by the high fluctuation of pigments between the different pigment pools.

Immediately after the photoinhibition experiments as well as after the recovery phase pigment extracts of the different photoadapted cultures were analysed and chlorophyll, carotenoid and xanthophyll amounts estimated (Fig. 2 and Table I). The Chl concentrations at the different stages of photoadaptation showed significant differences ranging from 18.8 mg/ml PCV in the low light adapted culture, to the 8.99 mg/ml in the medium light adapted one and down to 8.83 mg/ml PCV in the high light adapted cells, Chl concentrations paralleling the differentiation of the three cultures as monitored by the measurements of the photosynthetic activity (Fig. 1). The results in Fig. 1, as well as the alterations in the Chl content in dependence of their adaptation, documented in Fig. 2 and Table I suggest that the cultures exhibit the highest level of photoadaptation which can be reached under the respective experimental conditions. Significant differences between cultures

Table I. Chlorophyll, xanthophyll and total carotenoid content during photoadaptation (control), photoinhibition and after reversal to the initial light conditions (recovery). The experiments were performed with the unicellular green alga *Scenedesmus obliquus* and done, at least, in triplicate.

Pretreatment: low light intensity (5 Wm^{-2})

Samples	Control	Photo-inhibition	Recovery
Chlorophylls (mg/ml PCV)	18.80	18.80	18.70
Xanthophylls (mg/ml PCV)	3.24	3.01	2.94
Carotenoids (mg/ml PCV)	4.42	4.23	3.97

Pretreatment: medium light intensity (20 Wm^{-2})

Samples	Control	Photo-inhibition	Recovery
Chlorophylls (mg/ml PCV)	8.99	8.48	8.35
Xanthophylls (mg/ml PCV)	1.80	1.74	1.58
Carotenoids (mg/ml PCV)	2.45	2.92	2.04

Pretreatment: high light intensity (50 Wm^{-2})

Samples	Control	Photo-inhibition	Recovery
Chlorophylls (mg/ml PCV)	8.83	7.93	7.79
Xanthophylls (mg/ml PCV)	1.46	1.32	1.48
Carotenoids (mg/ml PCV)	1.82	1.51	1.71

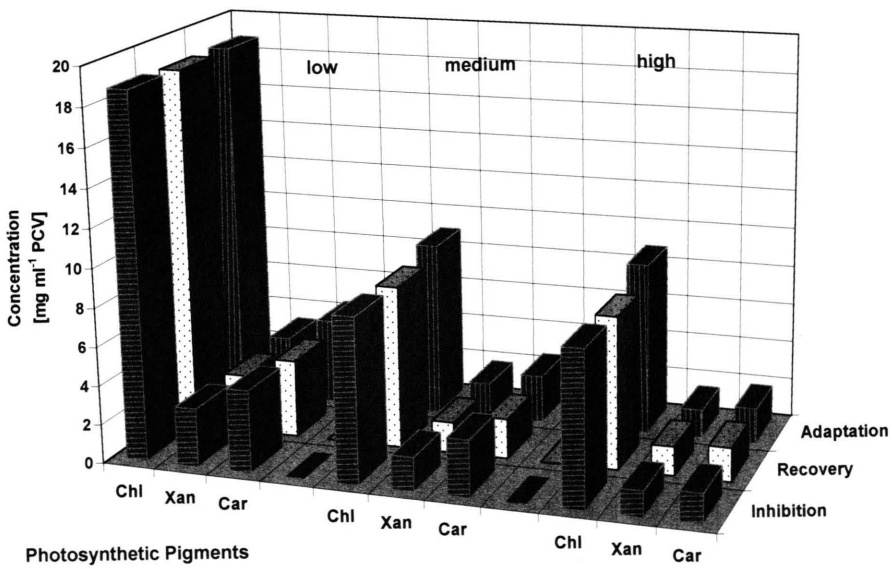

Fig. 2. Concentrations of chlorophylls (Chl), xanthophylls (Xan) and total carotenoids (Car) of cultures of *Scenedesmus* pre-adapted in low, medium and high light intensity, after adaptation, photoinhibitory and after reversal to the initial light conditions.

adapted to low and to medium light intensity are, on the one hand, present in the amounts of total carotenoids and total xanthophylls. On the other hand, cultures adapted to medium and high light intensities show smaller differences and thus support the above conclusion. Despite of the distinct differences in the levels of total Chls, total carotenoids and total xanthophylls in the photoadaptation experiments, the pigment concentrations remain constant with only very slight fluctuations during photoinhibition and also after reversal to the initial light conditions (Fig. 2 and Table I). HPLC analysis of the above described extracts and the quantification of the main peaks of their HPLC elution profiles showed, in contrast, significant differences in the amounts of the separated pigments (Fig. 3). A very interesting aspect in this context are the pigment changes observed in the violaxanthin/zeaxanthin cycle (vio/zea), but also the lutein/loroxanthin (lut/loro) ratio, since these pigments show the most prominent changes. The vio/zea-ratio decreased dramatically during the photoinhibition process and increased again on recovery to the initial light conditions (Table II). The cultures readapted at 20 and 50 Wm^{-2} show after

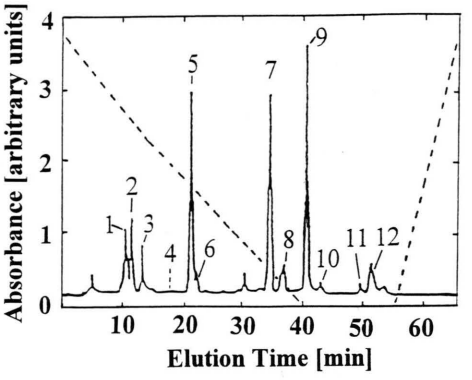

Fig. 3. A typical elution profile of chlorophylls and carotenoids as obtained from pigment extracts of *Scenedesmus* cultures adapted to different light conditions. The dashed line in the diagram indicates the water gradient superimposed on the solvent system. The numbers in the diagram stand for: **1** = Neoxanthin (10.5); **2** = Loroxanthin (11.5); **3** = Violaxanthin (13.2); **4** = Antheraxanthin (17.8); **5** = Lutein (21.5); **6** = Zeaxanthin (22.0); **7** = Chl b (34.5); **8** = Chl b' (36.8); **9** = Chl a (40.5); **10** = Chl a' (43.0); **11** = α-Carotene (49.5); **12** = β-Carotene (51.7). The numbers in parentheses after the compound name represent their mean elution times. The usual deviation is less than ± 0.2 min. The detection wavelength was 445 nm.

Table II. The intracellular level of violaxanthin (vio), zeaxanthin (zea), lutein (lut) and loroxanthin (loro) and the ratios vio/zea and loro/lut during photoadaptation (control), photoinhibition and after reversal to the initial light conditions (recovery). The experiments were done, at least, in triplicate.

Pretreatment: low light intensity (5 Wm^{-2})

Samples	Control	Photo-inhibition	Recovery
Violaxanthin (mg/ml PCV)	0.423	0.182	0.346
Zeaxanthin (mg/ml PCV)	0.139	0.148	0.149
Vio/Zea	3.040	1.230	2.320
Lutein (mg/ml PCV)	1.118	1.198	1.159
Loroxanthin (mg/ml PCV)	0.795	0.784	0.662
Loro/Lut	0.711	0.090	0.170

Pretreatment: medium light intensity (20 Wm^{-2})

Samples	Control	Photo-inhibition	Recovery
Violaxanthin (mg/ml PCV)	0.230	0.094	0.230
Zeaxanthin (mg/ml PCV)	0.098	0.210	0.057
Vio/Zea	2.350	0.450	4.035
Lutein (mg/ml PCV)	0.955	1.046	0.869
Loroxanthin (mg/ml PCV)	0.214	0.094	0.148
Loro/Lut	0.224	0.090	0.170

Pretreatment: high light intensity (50 Wm^{-2})

Samples	Control	Photo-inhibition	Recovery
Violaxanthin (mg/ml PCV)	0.167	0.023	0.165
Zeaxanthin (mg/ml PCV)	0.250	0.137	0.232
Vio/Zea	0.670	0.168	0.711
Lutein (mg/ml PCV)	0.640	0.641	0.718
Loroxanthin (mg/ml PCV)	0.346	0.270	0.284
Loro/Lut	0.540	0.421	0.395

their recovery pigment concentrations higher than the initial ones, whereas the cultures readapted at 5 Wm^{-2}, although there is an increase, do not reach the initial pigment contents (Table II). This shows that the low-light adapted cultures are not as capable of readaptation as those grown at 20 and 50 Wm^{-2} after the photoinhibition treatment. At the different levels of photoadaptation lutein is linearly reduced with the increase of light intensity, whereas the decrease of loroxanthin shows a minimum at 20 Wm^{-2}. While the behaviour of these two xanthophylls is commonly observed during photoadaptation, the increase of the lutein content during photoinhibition accompanied by a simultaneous reduction of loroxanthin is some-

thing new (Table II). During the recovery from photoinhibition a decrease is observed in the lutein contents, whereas an increase in loroxanthin levels was measured. The conversion of lutein into loroxanthin and violaxanthin into zeaxanthin, and *vice versa*, is correlated to photoadaptation, as well as to photoinhibition. This reconfirms the discrimination between photoinhibition and photoadaptation and indicates that the two xanthophyll cycles are representative for the general situation of the photosynthetic apparatus. It is obvious that the xanthophyll cycle with zeaxanthin, antheraxanthin and violaxanthin also functions in the unicellular green alga *Scenedesmus obliquus* under certain light conditions as described for higher plants (Goodwin, 1980). Photoadaptation under medium light intensities leads to a reduction of the levels of violaxanthin and zeaxanthin in comparison to their levels in low light, however, the decrease does not follow the same kinitics. An explanation for this phenomenon could be the reduction of the antennae under high light intensities, since these xanthophylls are found both, in the proximal and distal antennae systems (Siefermann-Harms, 1985). In contrast to low and medium light conditions, during photoadaptation at high light intensities (50 Wm^{-2}) an inversion of the biosynthetic pathway from violaxanthin to zeaxanthin takes place, possibly indicating that the limits of photoadaptation have been exceeded and that the cells are thus under stress conditions. The xanthophyll cycle normally only functions at photoinhibitory conditions, as demonstrated with cultures preadapted at low, medium and also at high light intensities. Analogous or similar observations were made with the lutein/loroxanthin cycle, meaning that parallel changes of both, the lut/loro and the vio/zea ratio were measured under the described photoadaptive conditions (Table II). This has to be interpreted as correlated changes on the level of the photosynthetic antennae, since at least lutein is required for the development of the LHCs and the reaction center of PS II (Humbeck *et al.*, 1989; Senger *et al.*, 1993; Humbeck and Bishop, 1986). Under photoinhibitory conditions the direction of the cycle is reversed (loro→lut; vio→zea). Knowing in which direction these two xanthophyll cycles work under the different applied conditions it is possible to distinguish photoinhibition from photoadaptation and to roughly determine their limits. In addition, a measure for the grade of photoadaptation and photoinhibition can be derived.

While photoadaptation takes place under physiological conditions, photoinhibition is a stress situation. It is known that polyamines are synthesized as stress factors in a great variety of environmental stress situations, e.g. osmotic and low temperature stress (Smith, 1985). Therefore the estimation of the intracellular polyamine content can be used as a measure for physiological situation of the culture. It can be expected that a stress situation like photoinhibition, in contrast to photoadaptation conditions, will cause an increase in the intracellular level of polyamines. The participation of polyamines in the assembly of the photosynthetic apparatus (Kotzabasis *et al.*, 1993b) and the involvement of polyamines in photosynthetic activity (Kotzabasis and Senger, 1994) and chloroplast photodevelopment (Andreadakis and Kotzabasis, 1996) suggested to examine the changes of the intracellular polyamine levels during photoadaptation and photoinhibition. The three cultures which had been adapted in white light of 5, 20 and 50 Wm^{-2} and had already been used for pigment analysis were also employed for the quantitative determination of the polyamine content of the cells. Those cultures which had been adapted in low and medium light intensity did not exhibit significant differences in their total polyamine content, whereas the cultures which had been adapted to the high light intensity showed a remarkable increase in polyamines (Table III). A similar behavior was also observed for the putrescine (Put)amount. The increase of the total polyamines, and here especially that of putrescine, is explained by the "abnormal" light conditions which were perceived by the cultures as stress. A similar increase in Put is also found under various other stress conditions (Dondini *et al.*, 1994). From these data optimum conditions for photoadaptation can be derived. After transfer of the differently photoadaptated cells to photoinhibitory conditions an increase in the polyamine level was observed which was reversed on return to the initial conditions (Table III). Regarding the increase of Put which is characteristic within a series of stress conditions (Dondini *et al.*, 1994) it was found that there was not only a simple increase of Put during photoinhibition, but that the increase was inversely proportional to the light intensity during

Table III. The intracellular level of putrescine (Put), spermidine (Spd) and norspermidine (NorSpd) during photoadaptation (control), photoinhibition and reversalt to the initial light conditions in the unicellular green alga *Scenedesmus obliquus*. The experiments were done, at least, in triplicate.

Pretreatment: low light intensity (5 Wm^{-2})

Samples	Control	Photo-inhibition	Recovery
Put (nmol/ml PCV)	2159	2593	2020
Spd (nmol/ml PCV)	1053	970	1490
NorSpd (nmol/ml PCV)	1926	2400	2780
Total polyamines (nmol/ml PCV)	5138	5963	6290

Pretreatment: medium light intensity (20 Wm^{-2})

Samples	Control	Photo-inhibition	Recovery
Put (nmol/ml PCV)	1889	2164	1975
Spd (nmol/ml PCV)	1216	1419	1098
NorSpd (nmol/ml PCV)	2481	3239	1912
Total polyamines (nmol/ml PCV)	5586	6822	4985

Pretreatment: high light intensity (50 Wm^{-2})

Samples	Control	Photo-inhibition	Recovery
Put (nmol/ml PCV)	3312	3626	2544
Spd (nmol/ml PCV)	575	628	458
NorSpd (nmol/ml PCV)	3863	4274	3928
Total polyamines (nmol/ml PCV)	7750	8528	6930

adaptation of the culture. In cells adapted at low light Put was 20.10%, in medium light adapted cells 14.55%, and in those pretreated with high light 9.48% higher during photoinhibitory conditions than in those cells which were kept under photoadaptive light conditions after the pre-treatment. The Put level is thus a measure for the intensity of the stress exerted on the respective culture during photoinhibition.

The reported results show that both xanthophyll cycles, the violaxanthin/zeaxanthin and the loroxanthin/lutein cycle, as well as the intracellular level of polyamines, especially of that of Put, are reliable biochemical parameters to determine the degree of stress exerted on *Scenedesmus*. They can thus serve as a tool to determine the limits between photoadaptation and photoinhibition and, furthermore, to estimate the extent of the two phenomena. Furthermore, it turned out that the photoinhibitory effect was inversely proportional to the status of pre-photoadaptation of a culture.

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