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Analysis of photosynthetic responses and adaptation to nitrogen starvation in *Chlorella* using *in vivo* chlorophyll fluorescence

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Abstract

Nitrogen (N) starvation resulted in degreening, inhibition of photosynthetic oxygen evolution and dark respiration, reduced survival, and increased age-specific mortality in both *Chlorella fusca* and *Chlorella vulgaris*. Analysis of *in vivo* chlorophyll (Chl) fluorescence induction kinetics revealed the presence of N-starvation-induced changes at the level of degreened thylakoids in both species. These changes included decreased yield of the photochemistry of photosystem 2 (PS2), and a declined photosynthetic efficiency. Synthesis of secondary carotenoids represented a biochemical change in carotenogenesis that had a photoprotective effect in degreened *C. fusca*. This inferred photoprotection was reflected in the delayed inhibition of oxygen evolution and improved survival of *C. fusca* under N-starvation. The effect was further elucidated by comparison with *C. vulgaris* which was not able to synthesize secondary carotenoids under the same conditions.

Additional key words: carotenoids; oxygen evolution; photosystem 2; species differences; respiration.

Introduction

Nitrogen starvation induces degreening of algal cells, decrease in amounts of N and carbon, and decrease in carbon/N and carbon/Chl ratios (Morris 1980, Darley 1982). N-starvation also reduces photosynthetic oxygen evolution in *C. fusca* (Grimme and Porra 1974, Sayed and Hegazy 1992). N-starved *C. fusca* develops non-appressed chloroplast thylakoids (Pyliotis *et al.* 1975), and acquires an orange colour due to synthesis of the secondary carotenoids echinenone and canthaxanthin (Sayed and Hegazy 1992). Synthesis of secondary carotenoids in N-starved *C. fusca* is important for improved survival under N-starvation (Sayed and Hegazy 1992).

However, work was needed to further assess the importance of secondary carotenoids for survival under N-starvation. In this respect the validity and versatility

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of application have made Chl fluorescence a powerful intrinsic tool in the study of thylakoid membrane function under stress (Samuelsson and Öquist 1977, Hetherington and Smillie 1984, Gilmour *et al.* 1985, Sayed *et al.* 1986, Krause and Weis 1991, Geider and Osborne 1992, Sayed 1994). The present paper deals with the effects of N-starvation on the photosynthetic machinery at the level of degreened thylakoid membranes in *C. fusca* and *C. vulgaris* (*Chlorococcales, Chlorophyta*). The work tries also to assess the importance of secondary carotenoid biosynthesis as an adaptive strategy to N-starvation using Chl fluorescence induction kinetics and demographic analysis.

Materials and methods

Algal cultures: *Chlorella fusca* 211-15 and *Chlorella vulgaris* Beij. (Algal Culture Collection, University of Göttingen, Germany) were grown in batch cultures using a mineral medium (Grimme and Porra 1974) at 25 °C and continuous irradiation of a bank of fluorescent lamps giving a photon flux density of 200 μmol m^{-2} s^{-1}. Nitrogen starvation was imposed by using a N-sparse medium (Grimme and Porra 1974). Cell numbers were determined using a haemocytometer chamber (*Bright-line, Reichert-Jung*, Buffalo, U.S.A.). Life table was constructed using demographic analysis including the parameters age interval in days (X-X'), length of interval when census was made (D_x), number of cells surviving to day X of each recording interval (N_x), survivorship (l_x) representing the probability of a cell of age zero surviving until the start of each recording interval, average mortality rate of the population (q_x) calculated by dividing the number of dying cells during the time interval by the number of individuals (N_x), and the killing power (k_x) between any pair of census dates calculated for each time interval as $\log N_x - \log N_{x+1}$ (Hutchings 1986).

Electron transport: Dark respiratory oxygen consumption and radiant energy-saturated photosynthetic oxygen evolution were polarographically measured at 25 °C in a Clark-type oxygen electrode (*Rank Brothers*, Cambridge, U.K.). Measurement of oxygen evolution was made at photon flux density of 800 μmol m^{-2} s^{-1}. Using spectroscopy techniques (*Lambda-3, Perkin-Elmer*, Connecticut, U.S.A.) total Chl ($a + b$) was determined (Bertrand and Schoefs 1997), and secondary carotenoids were determined using an extinction coefficient of 2200 (Chapman 1988).

Chl fluorescence: Samples containing approx. 1×10^6 cells per cm^3 were withdrawn from the cultures, and cells were separated from the medium by mild centrifugation at 1000×*g* for 5 min (*MLW, VEB Medizintechnik*, Leipzig, Germany). Separated cells were loaded on wet filter paper discs and dark adapted for 15 min. Chl fluorescence induction kinetics were then measured using a Plant Productivity Fluorometer (*SF 30, Richard Brancker*, Ottawa, Canada). Standard nomenclature of fluorescence parameters was followed (van Kooten and Snel 1990). Measured fluorescence parameters (Fig. 1) included the minimal fluorescence intensity (F_0), the peak fluorescence intensity (F_p), the maximal fluorescence intensity (F_m), and the steady-state fluorescence intensity (F_s). Calculated fluorescence parameters included the

variable fluorescence yield (F_v) defined as $F_v = F_p - F_0$ (van Kooten and Snel 1990), the half rise time $F_v/2$ (Bolhàr-Nordenkampf and Öquist 1993), and the ratios F_v/F_p (Butler and Kitajima 1975) and F_0/F_v (Kriedemann *et al.* 1985). Other calculated Chl fluorescence parameters included the fluorescence quenching (F_q) defined as $F_q = F_p/F_s-1$ (Briantais *et al.* 1979), and the Chl fluorescence decline ratio (Fdr) defined as Fdr = $(F_m) - (F_s/F_m)$ (Bolhàr-Nordenkampf and Öquist 1993). All measurements were routinely repeated at weekly intervals, results were presented as means, and standard error values were calculated.

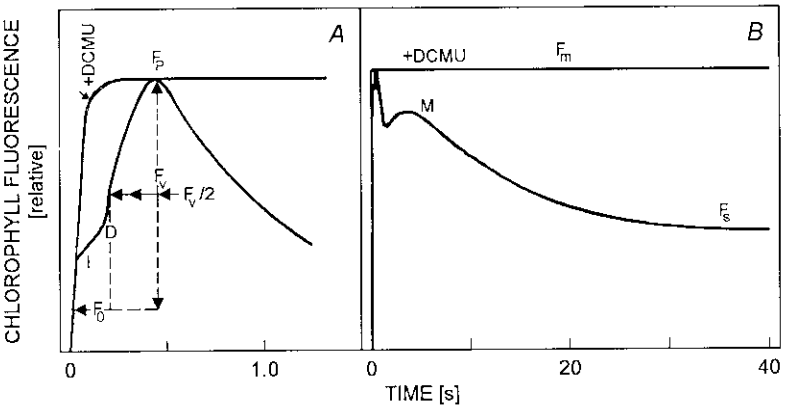

Fig. 1. Chlorophyll fluorescence kinetics of 10-min dark-adapted *Chlorella fusca*. (*A*) fast kinetics, (*B*) slow kinetics. Minimal fluorescence intensity (F_0), inflection level (I), dip (D), peak fluorescence intensity (F_p), variable fluorescence intensity (F_v), maximal fluorescence intensity (F_m), half rise time from F_0 to F_m ($F_v/2$), secondary peak (M), and steady-state fluorescence intensity (F_s).

Results

Imposed N-starvation resulted in a marked decline of the total Chl ($a \cdot b$) content in both *C. fusca* and *C. vulgaris* (Fig. 2*A,C*), respectively. N-starvation also resulted in reduction of the rates of dark respiration and photosynthetic oxygen evolution in both *C. fusca* (Fig. 3*A,B*) and *C. vulgaris* (Fig. 3*D,E*). These effects were somewhat delayed in *C. fusca* than in *C. vulgaris* as indicated by the time within which Chl content and rates of respiration and oxygen evolution declined to 50 % of their original control values (arrows in Figs. 2*A,C*, 3*A,B,D,E*). Moreover, the content of secondary carotenoids of control *C. fusca* cells was significantly higher than that of *C. vulgaris* cells, and under N-starvation secondary carotenoids increased in *C. fusca* and decreased in *C. vulgaris* (Fig. 2*B,D*).

N-starvation-induced changes in Chl fluorescence kinetics included a progressive rise in the value of F_0/F_v in both *C. fusca* (Fig. 3*C*) and *C. vulgaris* (Fig. 3*F*), in the latter exhibiting significantly higher values. Under N-starvation both *C. fusca* and *C. vulgaris* showed also marked reduction of the values of F_v/F_p (Fig. 4*A,E*), $F_v/2$ (Fig. 4*B,F*), F_q (Fig. 4*C,G*), and Fdr (Fig. 4*D,H*). This decline of Chl fluorescence parameters was delayed in *C. fusca* in comparison with *C. vulgaris*.

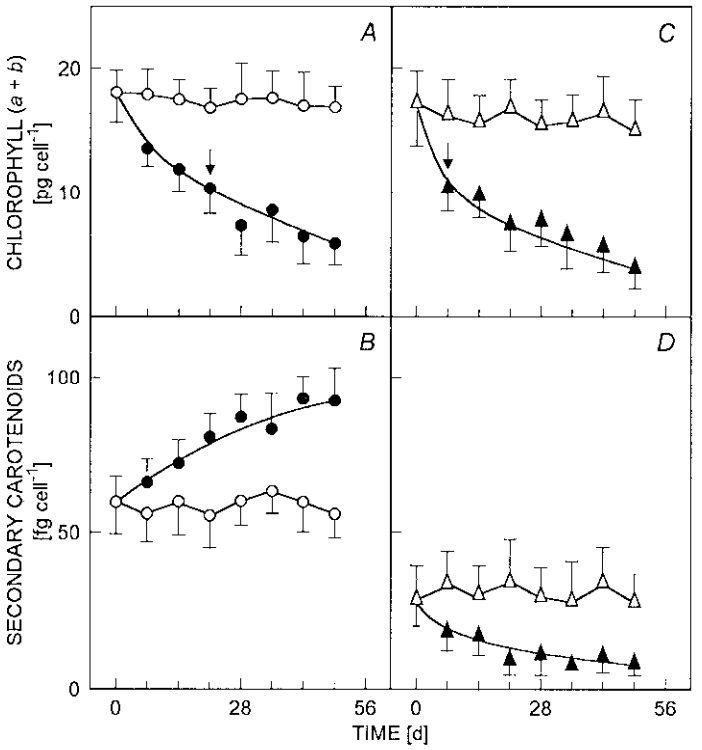

Fig. 2. Effect of nitrogen starvation on contents of chlorophyll ($a + b$) and secondary carotenoids in *Chlorella fusca* (*A,B*) and *Chlorella vulgaris* (*C,D*). Open symbols denote control cells, closed symbols denote N-starved cells, and arrows denote time within which parameters declined to 50 % of original control values (±SE, $n = 5$).

Demographic studies indicated that under N starvation *C. fusca* populations exhibited a higher survival (l_x) than *C. vulgaris* (Table 1). N-starvation resulted also in increased age-specific mortality (q_x) and killing power (k_x) in both *C. fusca* and *C. vulgaris*, the latter exhibiting remarkably higher values (Table 1).

Discussion

N-starvation resulted in degreening of both *C. fusca* and *C. vulgaris* as manifested by reduced Chl content (Fig. 2*A,C*). This degreening was reflected in the reduced rate of photosynthetic oxygen evolution in both species (Fig. 3*B,E*). Similar N-starvation-induced effects have already been reported in *C. fusca* (Grimme and Porra 1974, Sayed and Hegazy 1992). Moreover, both *C. fusca* and *C. vulgaris* have similarly responded to N-starvation by a general slow-down of metabolism manifested by a significantly reduced rate of dark respiration (Fig. 3*A,D*).

Despite these similarities, control *C. fusca* cells had a higher content of secondary carotenoids than control *C. vulgaris* cells, and were able to synthesize additional

amounts of these pigments under N-starvation (Fig. 2*B,D*). This induced secondary carotenoid biosynthesis is important for survival under N-starvation (Sayed and Hegazy 1992). Improved survival was perhaps reflected in the delayed inhibition of

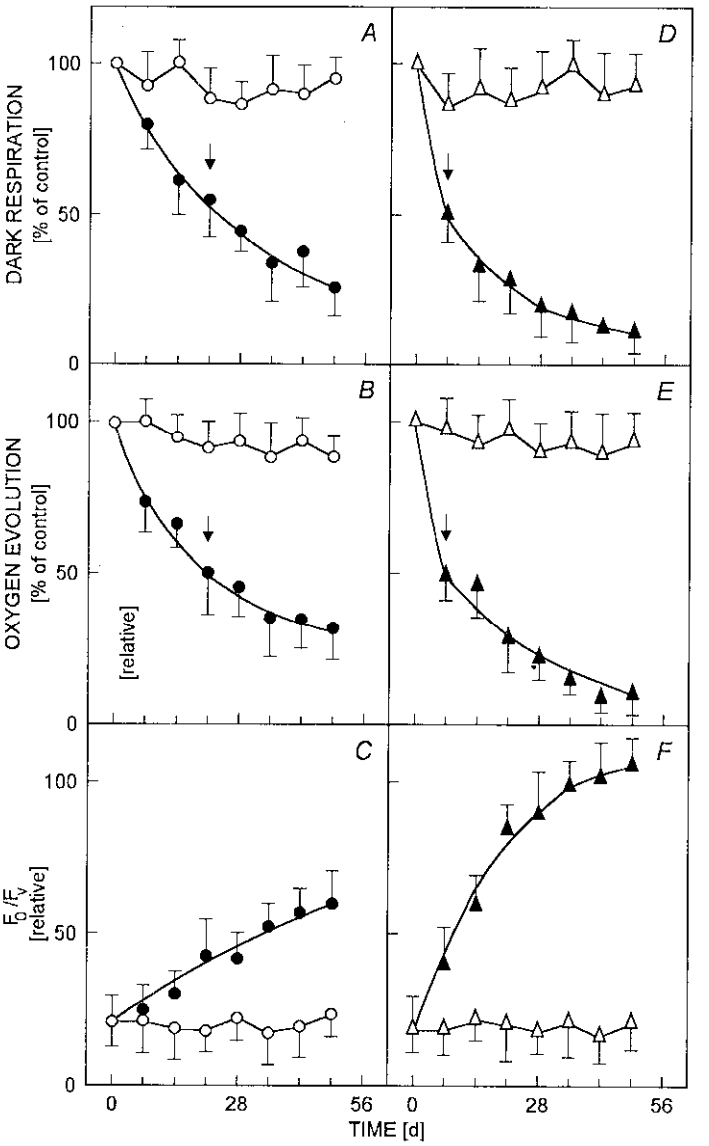

Fig. 3. Effect of nitrogen starvation on dark respiration, oxygen evolution, and F_0/F_v in *Chlorella fusca* (*A-C*) and *Chlorella vulgaris* (*D-F*). Control rates of dark respiratory oxygen consumption and photosynthetic oxygen evolution were 4.2 and 9.7 fmol cell^{-1} s^{-1}, respectively. Open symbols denote control cells, closed symbols denote nitrogen-starved cells, and arrows denote time within which parameters declined to 50 % of original control values (±SE, $n = 5$).

oxygen evolution, high survival, and low age-specific mortality of degreened *C. fusca* as compared with *C. vulgaris* which was incapable of secondary carotenoid synthesis (Fig. 3, Table 1).

Table 1. Life table of *Chlorella fusca* and *Chlorella vulgaris* under nitrogen starvation (-N). X-X' - age interval [d]; N_x - cells ($\times 10^4$) surviving to day X; lx - survival or probability of cell surviving to day X; q_x - average mortality rate per day during interval beginning with day X; k_x - killing power during interval beginning with day X. D_x, the length of interval was always 7 d.

X-X'		*C. fusca*				*C. vulgaris*			
		N_x	l_x	q_x	k_x	N_x	l_x	q_x	k_x
00-07	cont.	132	0.99	0.015	0.006	136	0.97	0.029	0.012
07-14		130	0.97	0.015	0.007	130	0.92	0.046	0.020
14-21		125	0.93	0.040	0.017	122	0.87	0.066	0.028
21-28		119	0.89	0.050	0.021	115	0.82	0.061	0.025
28-35		118	0.88	0.009	0.004	112	0.80	0.027	0.012
35-42		117	0.87	0.009	0.004	110	0.79	0.018	0.008
42-49		116	0.88	0.009	0.003	109	0.78	0.009	0.004
00-07	-N	107	0.97	0.028	0.012	120	0.92	0.083	0.035
07-14		68	0.62	0.574	0.197	65	0.50	0.846	0.266
14-21		60	0.55	0.133	0.055	52	0.40	0.250	0.097
21-28		55	0.50	0.091	0.038	46	0.35	0.130	0.053
28-35		52	0.47	0.058	0.024	41	0.32	0.122	0.051
35-42		50	0.46	0.040	0.017	35	0.27	0.114	0.047
42-49		49	0.45	0.020	0.009	33	0.25	0.061	0.025

This view is supported by analysis of Chl fluorescence kinetics. The value F_0/F_v increases abruptly at suboptimal levels of manganese, and is used for early diagnosis of Mn deficiency (Kriedemann *et al.* 1985). Moreover, the water-splitting apparatus of PS2 contains Mn (Andréasson and Vänngård 1988) and reversibly loses its oxygen-evolving capability under Mn deficiency (Gregory 1989). The observed increased values of F_0/F_v (Fig. 3*C,F*) were, therefore, indicative of N-starvation-induced changes at the water-splitting apparatus on the oxidizing side of PS2 in degreened thylakoids of *C. fusca* and *C. vulgaris*. Moreover, the values of F_v/F_p, F_q, and $F_v/2$ represent the maximum yield of photochemistry of PS2, the fluorescence quenching, and the size of the plastoquinone pool, respectively (Butler and Kitajima 1975, Briantais *et al.* 1979, Bolhàr-Nordenkampf and Öquist 1993). The recorded declined values of F_v/F_p, $F_v/2$, and F_q (Fig. 4) hence reflected induced changes at the reducing side of PS2 in degreened thylakoids of *C. fusca* and *C. vulgaris*. Furthermore, the value of Fdr is determined by the efficiency of the photosynthetic carbon reduction cycle (Lichtenthaler 1987, Lichtenthaler and Rinderle 1988, Bolhàr-Nordenkampf and Öquist 1993, Babani and Lichtenthaler 1996, Lichtenthaler and Miehé 1997), and the declined values of Fdr (Fig 4*D,H*) thus indicated low photosynthetic efficiency of N-starved cells of both *C. fusca* and *C. vulgaris*.

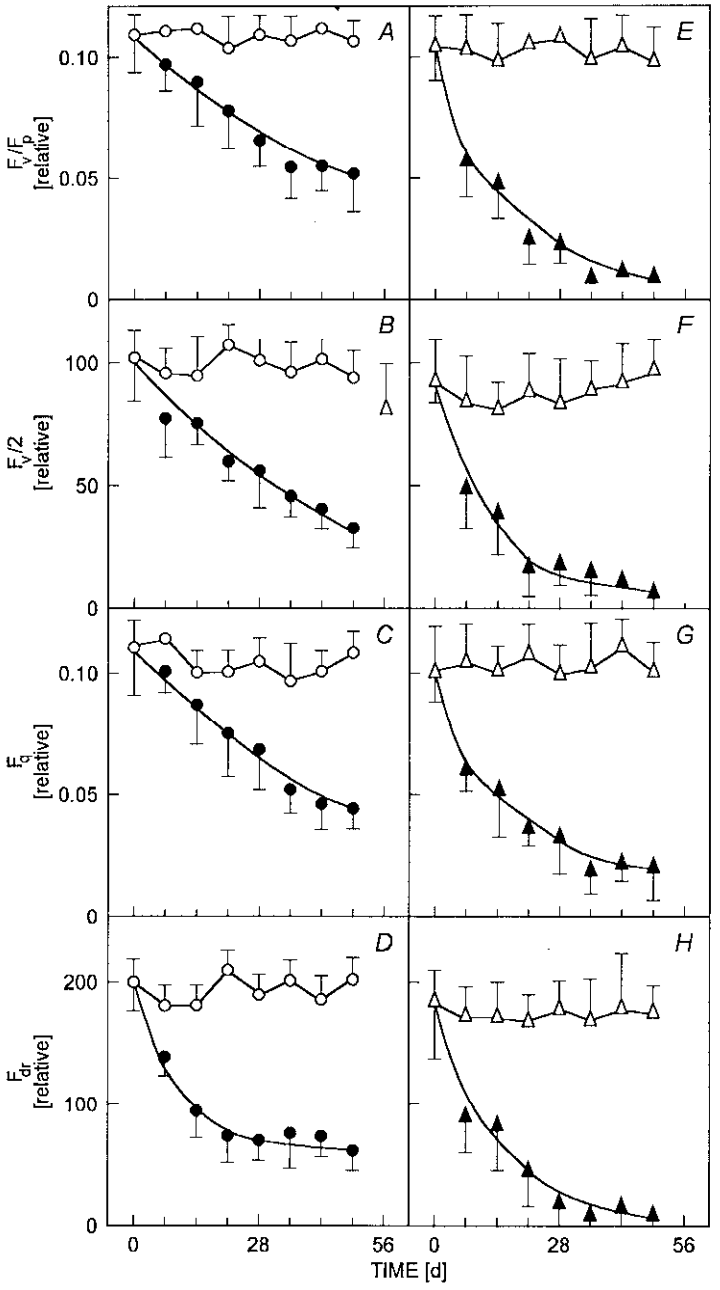

Fig. 4. Effect of nitrogen starvation on the parameters of chlorophyll fluorescence kinetics F_v/F_p, $F_v/2$, F_q, and Fdr in *Chlorella fusca* (*A-D*) and *Chlorella vulgaris* (*E-H*). Open symbols denote control cells, and closed symbols denote nitrogen-starved cells (±SE, $n = 5$).

It can, therefore, be concluded that N-starvation had profound effects on thylakoid membranes including degreening and adverse changes at the level of the photosynthetic electron transport chain in the vicinity of PS2. These changes had detrimental consequences for degreened cells including reduced photosynthetic efficiency, decreased survival, and increased mortality. The ability of *C. fusca* to synthesize secondary carotenoids may be an important photoprotective strategy for improved thylakoid membrane integrity, delayed inhibiton of photosynthesis, and hence for a better survival under N-starvation.

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