

Effects of Illumination and Nitrogen Starvation on Accumulation of Arachidonic Acid by the Microalga *Parietochloris incisa*

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Abstract—*Parietochloris incisa* is a unicellular freshwater green alga capable of accumulating high amounts of the valuable long-chain polyunsaturated arachidonic acid (AA) in triacylglycerols (TAG) of cytoplasmic oil bodies. To find the cultivation conditions providing maximum AA yield, the effects of illumination and N-availability on the dry weight (DW), chlorophyll, carotenoid, and AA content were studied. Under nitrogen starvation, TAG accounted for over 30% of dry weight (DW) and the AA content became as high as about 55% of total fatty acids. For biomass accumulation, light intensity of ca 400 $\mu\text{E m}^{-2} \text{s}^{-1}$ was found to be optimal for growing *P. incisa* on a complete medium. Lower light intensities (or a higher cell density of inoculum) resulted in a higher AA yield when the alga was cultivated on nitrogen-free media. In the absence of nitrogen, algal cells were unable to cope with high illumination and suffered from photooxidative damage, whereas the nutrient-sufficient culture survived under such illumination conditions, probably due to accumulation of carotenoids. Nitrogen-deprived *P. incisa* cells displayed elevated sensitivity to light.

Abbreviations: AA—arachidonic acid, FA—fatty acids, Car—carotenoids, PUFA—polyunsaturated fatty acids, TAG—triacylglycerols, Chl—chlorophyll(s).

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INTRODUCTION

Polyunsaturated fatty acids (PUFA), the vitamins of F groups, are important dietary components of animals and man. Among them, especially important are long-chain (C20) fatty acids (FA) of $\omega 6$ series. In particular, arachidonic acid (AA, 20:4 $\omega 6$) as a component of membranes, phospholipids of neurons of the brain. It is a precursor of physiologically active substances such as leukotrienes and prostaglandins (Hansen et al., 1997). Its positive influence on development of pre-term infants was demonstrated (Koletzko and Brown, 1991). Therefore, the capacity of some algae for synthesis of PUFA, AA in particular, may be important in the applied aspect (Cohen, 1990, 1999; Bigogno et al., 2002).

In most photosynthesizing microalgae, PUFA are present mainly in polar phospho- and galactolipids of chloroplast membranes whose composition is rather conservative (Thompson, 1996; Cohen, 1999). However, under certain conditions some unicellular algae are capable of inducing the synthesis of neutral lipids as triacylglycerols (TAG). As a result, fatty acids are accumulated in large quantities (Shifrin and Chisholm, 1981). Long-chain PUFA of $\omega 3$ -series are frequently present in cells of unicellular algae, while $\omega 6$ -FA occur much rarer and their content is as a rule significantly lower (Cohen et al., 1992). In particular, AA is not found in

significant amounts in lipids of most freshwater and marine green algae (Bigogno et al., 2002).

The unicellular freshwater alga *Parietochloris incisa* comb. nov. (Chlorophyta, Trebouxiophyceae) (Watanabe et al., 1996) is able to accumulate high quantities of TAG enriched in PUFA in cytoplasmic lipid globules (so-called "oil bodies"). It is important that AA dominates in FA of *P. incisa*: its content attains 33.6 and 42.5% of total FA at the logarithmic and stationary growth phases, respectively. Biosynthesis of lipids increases under unfavorable conditions retarding growth of the culture. Thus, under nitrogen starvation TAG account for up to 30% of the dry weight of cells with AA comprising up to 60% of total FA. Comparison with other algae shows that this organism is one of most rich natural sources of AA (Bigogno et al., 2002; Khozin-Goldberg et al., 2002; Merzlyak et al., 2007). Previous experiments demonstrated that optimization of the culture conditions of *P. incisa* for obtaining maximum production of AA and biomass is not trivial. In part, this is related to the fact that at low illumination the growth of the culture is retarded. At a high illumination, photodamage is frequent, especially under nitrogen starvation (Cheng-Wu et al., 2002).

In the present study, the influence of the intensity of illumination and the presence of nitrogen in the medium on the accumulation of biomass, FA, and AA

Table 1. The experimental design

Culture conditions	Variant					
	LL* + N	ML + N	HL + N	LL - N	ML - N	HL - N
Presence of nitrogen in the medium	+	+	+	-	-	-
Illumination, $\mu\text{E}/(\text{m}^2 \text{ s})$	35	200	400	35	200	400

* Designations of levels of illumination: LL—low illumination; ML—medium illumination; HL—high illumination.

in a culture of *P. incisa* is investigated for elucidation of conditions favoring accumulation of AA.

MATERIAL AND METHODS

Culture conditions. A strain of *P. incisa* isolated from the slope of Tateyama Mountain in Japan (Watanabe et al., 1996) was grown on a complete medium (+N) and a nitrogen-free (-N) medium BG-11 (Stanier et al., 1971) in glass columns at constant illumination by luminescent daylight lamps at three different light intensities (Table 1). A mixture of CO₂ and air (1 : 99) was bubbled through the cultures. The temperature was maintained at 25°. The initial content of chlorophyll (Chl) was 30 mg/l in all variants. The inoculum culture was diluted every day to support its logarithmic growth. Nitrogen starvation was conducted in the following way. The cells precipitated by centrifugation were washed with sterile distilled water three times and resuspended in the nitrogen-free medium BG-11. Then they were grown under the aforementioned conditions.

Analysis of fatty acids and pigments. Lyophilized cells, extracts of lipids, or individual lipids were transmethylated by incubation with 2% H₂SO₄ in methanol for 1 h at 80°. As an internal standard, heptadecanoic acid (margaric acid) was added to the samples. Gas chromatography of methyl esters of FA was performed according to Cohen et al. (1992). Identification of methyl esters of FA was made by co-chromatography with pure substances (Sigma, USA) and by the equivalent length of the carbon backbone chain (Ackman, 1969). The content of Chl and carotenoids (Car) in chloroform extracts was determined spectrophotometrically (Wellburn, 1994). At the least, two biological replications were used in analysis and two analytical replications were made for each of them.

RESULTS

Effect of illumination and nitrogen starvation on production of biomass. Figure 1 shows that the growth rate (accumulation of biomass) depended both on the presence of nitrogen in the medium and on the intensity of illumination. In the culture grown at a high intensity of illumination on the complete medium (HL + N, Table 1), the most rapid growth was observed. Under such conditions up to the 14th day, the yield of biomass was maximal. In the cultures grown at lower illumination

(ML + N and LL + N), the accumulation of biomass was 2–2.5 times lower in comparison with HL + N.

In the absence of nitrogen, by the third day of the experiment, the culture grown at the maximum light intensity (HL - N) accumulated more biomass than ML - N and LL - N. After the 3d day of cultivation the HL - N culture ceased to grow, the maximum biomass was accumulated by the culture grown at medium illumination (ML - N). However, the production of biomass was lower than on the complete medium. Up to the 14th day in the culture HL - N, the symptoms of photooxidative damage were recorded: bleaching of pigments with a synchronous decrease of the content of Chl and Car and the appearance of a peak with the maximum at 410 nm in spectra of cell extracts (the data are not presented), for the products of photodestruction of Chl (Merzlyak et al., 1996). In short time the HL - N culture perished. At the same time, in cultures ML - N and LL - N, no considerable decrease in the content of Chl was recorded.

In all cultures grown at medium and high illumination, there was an increased ratio of Car and Chl, which was more expressed in cultures starved of nitrogen. On the complete medium, the content of Car was twice as high as on the nitrogen-free medium. Due to the lower content of Chl, the cultures grown on the nitrogen-free medium manifested a significantly higher Car/Chl ratio.

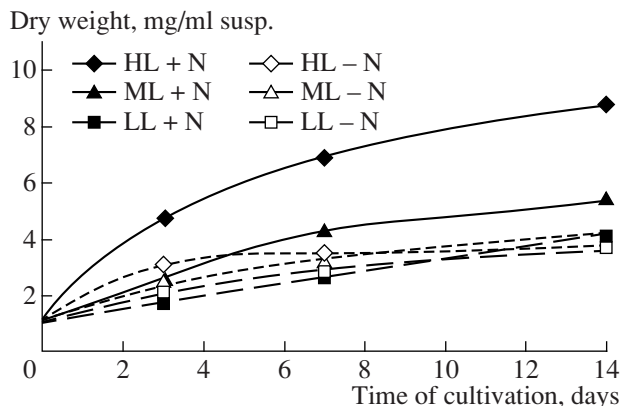


Fig. 1. Effect of illumination and nitrogen starvation on growth of a culture of *P. incisa*.

Table 2. Effect of nitrogen starvation and illumination on the composition and content (% of dry weight) of fatty acids in *P. incisa*

Variant	Days of cultivation	Content of fatty acids								AA	Total FA, % DW
		16 : 0	18 : 0	18 : 1 ω9	18 : 2	18 : 3 ω6	18 : 3 ω3	20 : 3 ω6	20 : 4 ω6		
HL + N	3	14.0	3.0	18.6	19.1	2.1	3.6	1.7	27.6	4.2	15.4
	7	9.7	2.6	22.3	15.6	1.1	1.7	1.5	36.5	9.0	24.6
	14	8.6	2.2	23.9	15.8	0.7	1.2	1.0	38.4	13.7	35.7
HL – N	3	11.4	2.0	25.0	12.5	1.6	1.6	1.1	36.5	6.8	18.6
	7	10.4	2.5	19.1	11.8	0.8	1.0	1.0	46.4	12.2	26.7
	14	9.5	2.2	14.5	10.9	0.7	0.7	0.9	52.2	15.1	28.9
ML + N	3	16.8	1.9	7.5	25.3	0.9	4.8	0.4	28.1	3.7	13.3
	7	15.2	2.3	7.7	23.7	1.1	2.7	0.7	32.2	3.7	11.7
	14	11.5	2.8	10.5	16.6	0.8	1.0	1.1	46.1	7.9	17.2
ML – N	3	13.2	2.4	11.0	13.3	1.6	2.1	1.4	44.4	6.7	15.1
	7	11.6	3.0	11.9	11.5	1.0	1.1	1.3	54.6	13.2	25.1
	14	10.4	2.7	11.1	9.7	0.7	0.7	1.2	56.5	19.0	33.5
LL + N	3	17.9	1.5	7.1	22.3	0.8	5.1	0.6	26.3	2.3	8.6
	7	16.8	1.7	7.7	23.3	0.5	3.5	0.5	24.9	2.2	8.7
	14	14.9	2.0	7.5	23.9	0.8	2.1	0.7	30.9	3.4	11.0
LL – N	3	13.5	2.4	7.3	17.7	1.7	2.9	0.9	41.4	5.3	12.8
	7	10.7	2.8	8.0	12.4	1.0	1.1	1.2	55.0	11.8	21.5
	14	10.4	2.5	7.9	10.7	0.7	0.8	1.2	58.2	14.9	25.6

Note: The content of total FA in the inoculum (0 days) was 8.0% of dry weight, including 1.8% AA.

Effect of illumination and nitrogen starvation on the composition of FA in P. incisa. According to chromatographic analysis (Table 2), the induction of synthesis of FA and lipids occurred not only under nitrogen starvation but also in the case of cultivation of *P. incisa* at a high illumination on the complete medium. Moreover, culture HL + N up to the 14th day had a maximum content of FA attaining 35.7% of the cell dry weight but the share of AA among them did not exceed 38.4% (13.7% of dry weight). The total content of FA in the culture ML + N was lower (17.2% of dry weight), almost half (45%) being AA.

In the absence of nitrogen, in all variants there was an increased content of FA in algal cells. The maximum content of total FA and AA (33.5 and 19.0% of dry weight, respectively) was in the culture ML – N. The content of FA in the culture HL – N increased only during the first seven days of cultivation and later on did not change. In the case of growth on the nitrogen-free medium at the minimum light intensity (LL – N), the proportion of AA in the total FA was maximal (58%). However, the absolute content of FA in this culture was comparatively low (25.6% of dry weight).

Comparison of the results of analysis of FA and pigments (Chl and Car) revealed that there is a linear relationship ($r^2 > 0.90$) between the content of AA and the

ratio Car/Chl in the cultures that were starved of nitrogen and grew at average and low illumination (Fig. 2). In the culture HL – N this relationship was also evident but followed a different trend due to an abrupt decrease in Chl after seven days of cultivation. Thus the ratio Car/Chl permits estimation of AA in cells of *P. incisa* starved of nitrogen growing at low and medium illumination (up to 200 $\mu\text{E}/(\text{m}^2 \text{s})$).

DISCUSSION

Unfavorable conditions and stresses, e.g., deficiency of mineral (nitrogen) nutrition with excessive intensive illumination, in some unicellular algae promotes biosynthesis of lipids. Lipids are assumed to be the sink for the excessive photoassimilates, which cannot be utilized in the absence of nitrogen for synthesis of protein and other compounds (Thompson, 1996). The formation of cytoplasmic oil bodies was reported to facilitate the synthesis of non-polar Car (usually β -carotene) or ketocarotenoids (e.g., astaxanthin). The Car accumulated within oil bodies are synthesized *de novo* or sequestered, probably together with lipids, from degrading thylakoid membranes (Mendoza et al., 1999). These Car seem to perform photoprotecting functions (Wang et al., 2003; Zhekisheva et al., 2002; Merzlyak et al., 2007) and protect lipids localized in

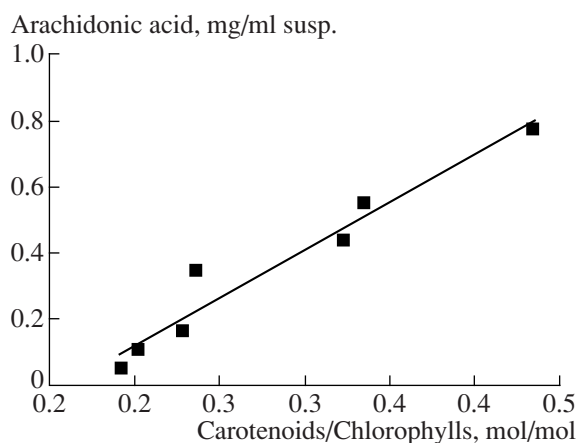


Fig. 2. Correlation between the ratio Car/Chl and the content of AA in *P. incisa* in the case of growth without nitrogen at an average and low illumination (NL – N and LL – N).

oil globules from photooxidation. Similar mechanisms have been discovered in some representatives of Chlorophyta such as *Dunaliella salina* (Mendoza et al., 1999) and *Haematococcus pluvialis* (Boussiba, 2000; Zhekisheva et al., 2002).

In *P. incisa*, under nitrogen deficiency conditions the synthesis of TAG containing high quantities of AA was also induced (Table 2, see also Khozin-Goldberg et al., 2002; Merzlyak et al., 2007). It is known that numerous oil bodies are also formed under nitrogen starvation (Merzlyak et al., 2007). In addition, high illumination promoted the synthesis of lipids even in the algae grown on the complete medium (Table 2). This agrees with the results of experiments on outdoor cultivation of *P. incisa* (Cheng-Wu et al., 2002). Under such conditions (complete medium and high illumination), the output of AA was doubled due to intensive growth and accumulation of biomass by the culture. It may be assumed that the mechanisms of adaptation to nitrogen deficiency and to intensive illumination are similar in *P. incisa* and in other green algae.

The obtained results demonstrate that, along with the presence of nitrogen in the medium, the intensity of illumination is an important factor controlling the quantitative and qualitative composition of lipids and the content of AA in *P. incisa*. Illumination about $400 \mu\text{E}/(\text{m}^2 \text{ s})$ is optimal for production of the maximum amounts of biomass with a sufficiently high content of AA in case of growth on the complete medium. Under nitrogen starvation, such illumination levels may cause photodamage of cells. This may be avoided, e.g., by use of an inoculum of higher density.

The obtained data also indicate that the cells starved of nitrogen are more susceptible to photodamage. It may be assumed that the limitation of protein synthesis by nitrogen deficiency impairs the functioning of systems of activated oxygen species detoxication and processes of reparation of the photosynthetic apparatus.

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