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Studies on the Impact of Nitrogen Starvation on the Photosynthetic Pigments Through Spectral Properties of the Cyanobacterium, *Spirulina platensis*: Identification of Target Phycobiliprotein under Nitrogen Chlorosis

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Abstract: Both macro and micro nutrients are essential for plant and algal growth and development. Limitation of nitrate in the medium, modulate the organization and functions of photosynthetic apparatus. In *Spirulina* lower concentrations (60, 40 and 20μM) of nitrate in medium results in the development of altered energy transfer between the photosynthetic pigments was proved by spectral properties. Particularly in phycobilisomes nitrogen stress causes alterations in the structural organization as well as changes in the energy transfer process under both *in vivo* and *in vitro* conditions. From the spectral data it supports that phycocyanin is the target pigment protein for the nitrogen chlorosis in the cyanobacterium, *Spirulina platensis*.

Key words: Nitrogen chlorosis · Phycobilisomes · Spectral alterations · *Spirulina platensis*

INTRODUCTION

The availability of inorganic nutrients influences and regulates the plant/ cyanobacterial growth and development. Nitrogen is a quantitatively important bioelement, which is incorporated into the biosphere through assimilation process carried out by the microorganisms and plants. In a variety of ecosystems the combined nitrogen supply limits growth and physiology of cyanobacteria. The diazotropic strains of cyanobacteria are able to fix nitrogen; thereby escaping from nitrogen depletion. In contrast, non-diazotropic cyanobacteria respond to the lack of nitrogen source by a process called bleaching of photosynthetic pigments [1]. This results in the change of colour of cultures from blue-green to yellow, a process known as chlorosis [2]. Even chlorosis also occurs upon starvation for other nutrients, which shows differences at cellular level depending on the nature of nutrient limitations [3, 4]. There is a suggestion that nitrogen starvation induces chlorosis and maintains low level of photosynthesis during nitrogen limitation [5].

Of the different chlorotic reactions, that include by nitrogen starvation has been most extensively studied. Since PS II of cyanobacteria contain phycobiliproteins (PBPs) as light harvesting complex [6], nitrogen stress can induce alterations in the LHC of cyanobacteria. As mentioned above phycobilisomes (PBsomes) can be readily degraded by proteases under nitrogen-starved conditions [7]. The pattern of degradation in *Synechococcus* 6301 consists of two phases [8]. First the PBsomes lose the PC hexamers and linkers located most distal to the core during trimming process which reduces 50% of the PBsome size. But under nitrogen stress both trimming of rods and PBsome core degradation also occurs in cyanobacteria [9].

Pbsome degradation requires energy, protein synthesis and unstable proteolytic activity [10]. A serine type protease capable of degrading PBPs as well as other protein has been reported by several workers [11]. A second type of proteolytic activity containing enzyme has been shown namely phycocyaninase involved in the degradation of PBPs under nitrogen deprivation [12]. Nitrogen starvation of *Synechococcus* 6301 causes the decrease of PC and linker polypeptides like 75, 33 and 30 kDa and affect the energy transfer from PBsomes to the P_{680}, RC of PS II. The second phase of response to nitrogen starvation is gradual loss of Chl *a* and in third phase the cells become depigmented and reside in dormant state. From this state they will be re-entering into growth with in few days, by the addition of nitrogen source. The material released by protein degradation may provide substances for the synthesis of new polypeptides required for acclimation to new nitrogen source [1].

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MATERIALS AND METHODS

Organism and Treatment: The mother culture of *Spirulina platensis* was obtained from National Facility for Blue green algal Collection, New Delhi, India and cultured autotropically in Zarrouk's medium [13] at 25±2°C under continuous illumination (20 Wm^{-2}). Log phase cells were collected by centrifugation at 5000 xg for 10 minutes, then they were suspended in the different low concentrations of nitrate containing medium (60, 40 and 20μM $NaNO_3$) for 24 hrs. After incubation cells were extracted and continued to further experiments. Intact cells equivalent of 6 μg of Chl were suspended in 3 ml of reaction buffer for measuring spectral properties.

Isolation of Pbsomes: Control and treated cells were grown up to mid-log phase in the medium were harvested by filtration on to a what No.1 filter paper using a Milipore filter assembly. PBsomes were isolated from *Spirulina* cells according to the method of Gantt *et al.* [14].

Spectral Measurements

Absorption Spectra of Intact Cells: After harvesting the cells, the pellets were suspended in 3 ml of reaction buffer. The reaction mixture contained reaction buffer 25 mM Hepes-NaOH (pH 7.5) and intact cells equivalent to 6 μg Chl *a*. This cell suspension was taken for scanning the absorption spectra from 400 nm to 750 nm. The absorption spectra of intact cell suspension were taken by using by using a Hitachi- 557 double beam spectrophotometer. At 750 nm the absorption of the cell suspension was adjusted to give approximately the same reading.

Fluorescence Emission and Excitation Spectra: Sample preparation was done in the same way as described above. The reaction mixture contained reaction buffer and the intact cells equivalent to 5 μg Chl *a*. The Chl fluorescence emission spectral measurements were done in a Perkin-Elmer fluorimeter (LS-5, England). The exciting beam was at 440 nm with a 5 nm slit width. The emission was collected from 600 nm to 750 nm with a slit width of 5 nm. Cells equivalent to 5μg of Chl *a* was used in all fluorimetric assays. Intact cells or PBsomes or pigment proteins were excited at 545 nm to excite specifically PC. The spectra were collected from 600 to 750 nm with 580 nm light beam. The spectra were collected from 600 to 750 nm with the above mentioned slit width. The spectra have not been corrected for the absolute spectral sensitivity of the fluorimeter.

RESULTS AND DISCUSSION

Cells grown in 200 μM nitrate containing medium exhibited no changes in growth characteristics. The stress being induced after incubation of cells under 60, 40 and 20 μM nitrate containing media. Therefore we have studied the effect of nitrogen stress on the spectral properties of the cyanobacterium, *Spirulina* under both *in vivo* and *in vitro* conditions to identify the target photosynthetic pigment under selected concentrations of nitrogen.

Figure 1 shows the absorption characteristics of control cells. The peak at 681 nm is due to the absorption of Chl *a* peak; at 621 nm is due to the absorption of PC of PBsomes; a hump at 480 nm is due to the absorption of carotenoids; and a peak at 437 nm is due to soret band of Chl *a* [15]. Nitrogen deprivation to 40 μM caused huge decrease in PC absorption by marginally effecting in the chlorophyll and carotenoid absorption (Fig. 1). In addition nitrogen stress caused red shift in the peak position by 3 nm in PC absorption. This decrease and alterations in PC absorption could be due to changes in the apoprotein and chromophore interaction. The effect was further characterized by calculating the absorption ratios of different pigment proteins such as A_{440}/A_{680}, A_{470}/A_{680} and A_{621}/A_{680} (Table 1).

In control cells the ratio of absorption at soret band region to the absorption at red region of chlorophyll was 1.16. Nitrogen stress from 60μM to 20μM did not bring any change in the ratio of chlorophyll absorption. The ratio of absorption at carotenoid region to the absorption at the red region of the chlorophyll was 0.89 in control cells (Table 1). The increase of nitrogen stress could not influence the ratio of carotenoids to chlorophyll absorption. This shows that nitrogen stress could not affect neither chlorophyll nor carotenoids. The ratio of absorption at PC region to the absorption at red region of the chlorophyll was 0.96 in control cells. Nitrogen stress brought gradual decrease in the ratio from 0.87 to 0.82 in 20 μM nitrate medium containing grown cells. This clearly demonstrates that PBPs are major targets among other pigment proteins.

Since nitrogen stress affected the PC absorption quite extensively, we have measured the room temperature PC fluorescence emission spectra of *Spirulina* cells which were grown with less nitrogen containing medium. In control cells excited at 545 nm, an emission peak at 647 nm emanating from PC was prominent in the spectrum (Fig. 2) [15]. Incubation of cells with 40 μM and 20 μM nitrogen

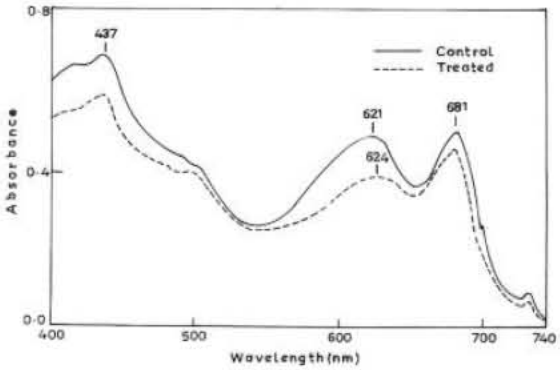

Fig. 1: Effect of nitrogen stress (40 μM) on the absorption spectra of the intact cells of the cyanobacterium, *Spirulina platensis*

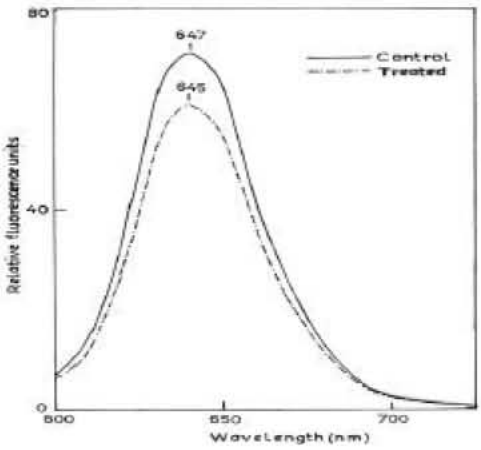

Fig. 2: Effect of nitrogen stress (60 μM) on PC fluorescence emission spectra of intact cells of *Spirulina*. Cells equivalent to 6μg of Chl *a* were used. Cells were excited with 545 nm light beam (Slit width for both excitation and emission was 5 nm)

containing medium exhibited decrease in the PC fluorescence emission and induced blue shift in the peak position by 2 nm (Fig. 2). Control cells exhibited 72 relative units of PC fluorescence with an emission peak at 647 nm. Nitrogen stress induction by the depletion of the nitrogen from 60 μM to 20 μM gradually brought the decrease from 14% to 40%. In addition 5 nm blue shift was noticed in the nitrogen stressed samples (Table 2). This clearly demonstrates that the nitrogen stress induced alteration in energy of energy transfer from PC to Chl *a* and structural changes of PBPs.

To further strengthen our argument the cells were excited with 440 nm light specially targeting Chl *a* present in the PS II. In control cells two peaks at 654 and 680 nm

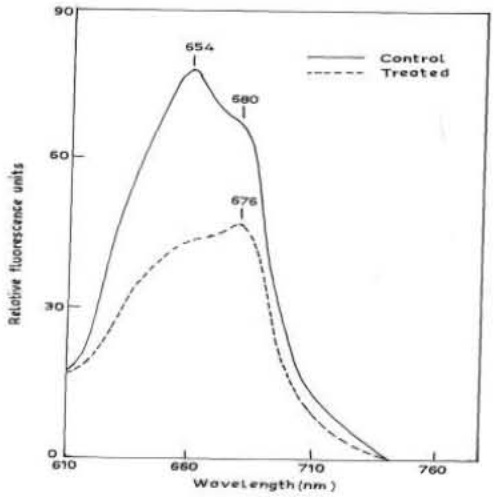

Fig. 3: Effect of nitrogen stress (40 μM) on fluorescence emission spectra of intact cells of *Spirulina*. Cells were excited with 440 nm. Cells equivalent to 6μg of Chl *a* were used (Slit width for both excitation and emission was 5nm)

were prominent in the spectrum. 654 nm peak is contributed by PC where as 680 nm is contributed by PS II Chl *a* (Fig. 3). Nitrogen stress (40 μM grown cells) caused 50% decrease in PC emission and 40% in Chl emission with a blue shift by 4 nm. This clearly confirms the structural alterations of Chl proteins. From these spectra it is clear that the energy is not flowing with 100% efficiency for PC to Chl *a*. Table 3 shows the ratio of Chl *a* to PC emission of control cells was 0.86. In treated sample the ratio has enhanced to 1.10 indicating the improper energy transfer in nitrogen depleted cells (40 μM). In cyanobacteria the PBPs constitute the major light harvesting pigment protein complexes, which are attached to the outer surface of the thylakoid membranes [16]. The light energy absorbed by PBsome is transferred to the PS II reaction center (RC) in the following sequence: PC→ APC→ Chl *a* [15].

A variety of environmental factors are known to affect the efficiency of energy transfer from PC to Chl *a* i.e., high temperature [17], low temperature [18]. From the *in vivo* results it is clear that nitrogen stress can influence the energy transfer from PC to Chl *a* in intact cells of *Spirulina*. To support the *in vivo* observations, we have also made an attempt to study the effect of nitrogen stress on PBsomes by isolating them by using sucrose density gradient. After removing the PBsomes from both control and treated samples sucrose has been removed by dialysis and the samples were used for spectral studies.

Table 1: Effect of different concentrations of nitrogen (60, 40 and 20 μM in growth media) on absorption properties of the intact cells of *Spirulina*. The values are average of 3 separate experiments

Concentration of $NaNO_3$ (μM)	Absorption ratio		
	440/680	470/680	621/680
Control	1.16	0.89	0.96
60	1.12	0.88	0.87
40	1.13	0.87	0.84
20	1.16	0.88	0.82

Table 2: Effect of different concentrations of nitrogen (60, 40 and 20 μM in growth media) on the PC fluorescence emission properties of the intact cells of *Spirulina* at room temperature. The values are average of 3 separate experiments

Concentration of $NaNO_3$ (μM)	Phycocyanin fluorescence emission		
	Intensity (Relative units)	Peak position	Percent decrease
Control	72	647	0
60	62	645	14
40	51	642	29
20	43	642	40

Table 3: Effect of different concentrations of nitrogen (60, 40 and 20 μM in growth media) on the Chl *a* fluorescence emission properties of the intact cells of *Spirulina* at room temperature. The values are average of 3 separate experiments

Sample	F_{680}	F_{654}	F_{680} /F_{654}
Control	90	105	0.86
Treated (40μM)	64	58	1.10

Table 4: Effect of different concentrations of nitrogen (60, 40 and 20 μM in growth media) on the PC absorption and fluorescence emission properties of the Pbsomes of *Spirulina* at room temperature. The values are average of 3 separate experiments

Concentration of $NaNO_3$ (μM)	Phycobilisomes			
	Absorption		Fluorescence emission	
	Optical density	Peak position, nm	Fluorescence emission intensity	Peak position, nm
Control	0.16	615	72	671
60	0.14	614	62	669
40	0.13	613	51	668
20	0.12	612	43	667

Table 4 shows the changes in the absorption properties and fluorescence emission characteristics of control and PBsomes of nitrogen stressed cell. Nitrogen stress brought 25% decrease in the absorption intensity and shifted the peak towards the blue region of the spectrum by 2 nm from 615 nm to 613 nm. Excitation of isolated Pbsomes by 545 nm brought 30% decrease in the fluorescence intensity at 670 nm. This clearly demonstrates that nitrogen stress is affecting the both absorption and energy transfer in PBsomes under nitrogen stress.

Thus spectral alterations of PBPs are partially responsible for the loss of PS II photochemistry in this cyanobacterium. These spectral properties are in agreement with the observations of Murthy and Mohanty [19]. Prolonged incubation under nitrogen stress induces serine type of protease capable of degrading the PBPs as reported by others [11]. The amino acids released by the protein degradation may provide substrates to meet the needs for the synthesis of new proteins for its survival under nitrogen stress [1].

This clearly demonstrates that nitrogen stress affects energy transfer and spectral properties under both *in vivo* and *in vitro* conditions which are responsible for the loss of PS II photochemistry in this cyanobacterium. In addition to that loss of energy transfer also indicating that PC seems to be the target for the nitrogen stress in the cyanobacterium, *Spirulina platensis*.

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