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Full Length Research Paper

Interplay between calcium influx and nitrate assimilation in *Spirulina platensis*

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Nitrate assimilation and its interplay with Ca^{2+} ion transport has been studied in *Spirulina platensis* PCC 7345, an alkaliphilic filamentous cyanobacterium. We have used verapamil, a calcium-channel blocker and sodium orthovanadate, a calcium efflux blocker, to study the effect of nitrate assimilatory setup. Nitrate uptake (NU) increased significantly with verapamil but was inhibited by sodium orthovanadate. Nitrate reductase and nitrite reductase activities were stimulated with verapamil as well as sodium orthovanadate. Glutamine synthetase activity was not significantly altered by both the inhibitors. Our results suggest that the steady state levels of NU, NR and NiR activities are inter linked with Ca^{2+} influx into the cells from the external medium.

Key words: *Spirulina*, nitrate uptake (NU), nitrate reductase (NR), nitrite reductase, glutamine synthetase.

INTRODUCTION

The nitrogen control and metabolism are well studied in several bacteria and higher plants (Lin and Stewart, 1998; Langendorfer et al., 1988). Each step in carbon and nitrogen assimilation is tightly regulated in response to environmental stimuli. The regulation involves various signal transduction systems adapted by these organisms (Merrick and Edwards, 1995; Ninfa et al., 2000). These signaling molecules have acquired a very little attention in non-nitrogen fixing *Spirulina platensis. Spirulina* is used as dietary supplement by natives in Africa, Japan and America, owing to its richness in proteins, carotenoids and other micronutrients. It also has the ability to modulate immune response and exhibits anti-inflammatory properties (Karkos et al., 2008). For these reasons studying the key biochemical signals in nitrate assimilatory pathway of *S. platensis* is of relevance for understanding its adaptations.

In cyanobacteria, the role of calcium ions [Ca^{2+}] as an intracellular second messenger is supported by a significant amount of literature (Smith and Wilkins, 1988; Pitta et al., 1997; Torrecilla et al., 2004). Ca^{2+} is an important factor regulating several cyanobacterial cellular processes. Intracellular free Ca^{2+} concentration increases several fold in heterocysts and is regulated by CcbP, a Ca^{2+} binding protein found in *Anabaena* (Shi et al., 2006). This Ca^{2+} mediated signaling has received a very little attention in nitrate assimilatory pathway of non-nitrogen fixing *Spirulina platensis.*

In nitrogen fixing organisms, several experimental evidence indicate that Ca^{2+} is not only playing a major role in heterocyst differentiation (Torrecilla et al., 2004), but also is required for the effective functioning of the photosynthetic apparatus (Brand et al., 1983; Becker and Brand, 1985). Ca^{2+} is reported to regulate the phototactic responses and motility in *Synechocystis* (Moon et al., 2004). In *S. platensis*, Ca^{2+} starvation has been reported to enhance NR activity and a decline in GS activity (Singh and Singh, 2000). Genes encoding many Ca^{2+}-binding domains have recently been identified in *S. platensis* genome (Fujisawa et al., 2010).

To scrutinize the regulation mediated by the Ca^{2+}, it is essential to quantitate the changes in intracellular Ca^{2+} levels in response to environmental stimuli. However, quantitation of Ca^{2+} levels during cellular signaling events has proven very difficult. However, these organisms respond to almost any kind of external stimulus altering their cytoplasmic free Ca^{2+} concentration (Berkelman et al., 1994). The fact that most of this Ca^{2+}-dependent

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processes responds to treatments which artificially alter the intracellular Ca^{2+} content gives us a means for studying Ca^{2+} ion mediated regulation (Smith and Wilkins, 1988).

Our group is involved in understanding the signal transduction events in nitrogen assimilatory pathway of *Spirulina*. In *S. platensis* PCC 7345, we have recently reported the up-regulation of a multi sensor histidine kinase (hstk), when NO^{3-} was added to nitrogen starved filaments (Logeswaran et al., 2011). This study was targeted to identify the interplay between Ca^{2+} ion transport and nitrate assimilatory pathway in *S. platensis*. In this study we have used Ca^{2+} ion channel inhibitors such as verapamil and sodium orthovanadate in nitrate assimilatory pathway of *S. platensis,* to distinguish the different levels of control (if any) posed by these signal transduction systems.

MATERIALS AND METHODS

Growth

S. platensis strain PCC 7345 obtained from the Pasteur Culture Collection was grown in Zarrouk's medium (pH 10.5) (Zarrouk, 1966). The exponential phase cultures were obtained by growing at 30 ± 2°C, under continuous white light illumination (4 Klux) for 8 to 9 days.

Experimental condition

Nitrate treatment was given before each enzyme assays. The treatments were done using exponentially growing cultures (9-day-old culture). 100 mL cultures were washed twice with ice-cold sodium bicarbonate buffer (10 mM; pH 10.5). The pellets were re-suspended in a nitrate free Zarrouk's medium for 48 h for NO_3^- depletion (Collier and Grossman, 1992) and further added 10 mM KNO_3 for NO_3^- repletion (El-Anwar et al., 1999), with/without inhibitors (25 to 100 µM verapamil and 0.2 to 1 mM sodium orthovanadate (Na_3VO_4)). The filaments were then lysed and enzyme extracts were prepared after every 30 min of addition of inhibitors for 2 h. The nitrate repleted filaments without the inhibitors were used as controls. All experiments were repeated thrice and the results are expressed as mean ± SE.

Preparation of crude extract for enzyme assay

For preparation of the enzyme extract, samples were taken from each flask at a regular interval of 30 min. Filaments were collected by centrifugation and resuspended in 20 mM Tris.Cl (pH 7.5) containing 200 mM NaCl and 10% glycerol for GS activity and in potassium phosphate buffer (pH 7.4) for NiR activity. The cell suspension was sonicated at 4°C for 5 min. (10 s on, 30 s off) at 18 KHz, using Microson ultrasonic cell disrupter. The sonicated sample was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was transferred into microfuge tube and used for glutamine synthetase (GS) assay and nitrite reductase (NiR) assay.

Protein estimation

Protein concentration was determined using Lowry's assay (Lowry, 1951). 400 µl of reagent D (prepared by mixing together 48 ml of 2% Na_2CO_3, 1 ml of 0.5% $CuSO_4.5H_2O$ and 1 ml of 1% Na-tartarate) was added to 100 µl of the crude extract, mixed well, and incubated at room temperature for 10 min. 100 µl of the Folin-Ciocalteus reagent diluted with water 1:1 was then added. After 30 min of incubation, the absorbance at 650 nm was recorded using JASCO V-630 UV/Vis spectrophotometer. Bovine serum albumin (BSA) was used as a standard in the range 50 – 200 µg.

Nitrate uptake assay

Nitrate uptake rate was assayed by measuring nitrate depletion from the external medium (Bartzatt and Donigan, 2004). To 100 µl of spent medium, 450 µl diphenylamine reagent and 425 µl of 18 M H_2SO_4 were added. Optical density was measured at 600 nm using JASCO V-630 UV/Vis spectrophotometer. The concentration of nitrate ions was determined using potassium nitrate as standard.

Nitrate reductase assay

Nitrate reductase (NR) activity was measured using the method described by Zhihui and Guolan (2000). The method of Zhu (1990) was used to determine the nitrite concentration in the test solution. Each test (1 ml) solution was added to 2 ml 15% HCl containing 1% sulfanilamide and 2 ml 15% HCl containing 0.02% N-1-naphtylethylendiamine dehydrochloride. Absorbance of the above solution was measured after 30 min at 520 nm. The amount of nitrite formed per mg of protein was determined using standard curve obtained using known concentrations of sodium nitrite.

Nitrite reductase assay

Nitrite reductase (NiR) activity was assayed using the method described by Losada and Paneque (1971). The 100 µl of extract was incubated in a solution containing 0.4 mL of 0.1 M potassium phosphate buffer (pH 7.4), 100 µl of 15 mM sodium nitrite, 200 µl of 5 mM methyl viologen, and 200 µl of 86.15 mM sodium dithionite in 190 mM sodium bicarbonate. The reaction was stopped by a violent agitation on vortex. Nitrite ions were assayed using 2 mL of 15% HCl containing 1% sulfanilamide and 2 mL of 0.02% solution of N-1-naphtylethylendiamine dehydrochloride. The amount of nitrite reduced per µg of protein was calculated from a standard curve plotted using the OD_{540} values obtained from known amounts of nitrite.

Glutamine synthetase assay

Glutamine synthetase (GS) activity was assayed using the method described by Robinson et al., (2001). The synthetic GS assay mix (0.5 ml) consisted of 50 mM L-glutamate, 55 mM $MgCl_2$, 46 mM hydroxylamine-HCl, and 92 mM imidazole, pH 7.0. The reaction was initiated by the addition of ATP at a final concentration of 20 mM. Blanks were run in parallel, substituting water for ATP. All reactions were stopped with 1 ml of an acidic $FeCl_3$ solution (55 g of $FeCl_3$, 20 g of trichloroacetic acid, and 21 ml of HCl per liter of solution). The formation of γ-glutamyl hydroxamate-iron complex (*γ*GH-Fe) was quantified at 540 nm. GS activities were calculated by normalizing with protein concentration determined via Lowry's method.

Statistical analysis

NU, NR, NiR and GS activities presented in this work are the

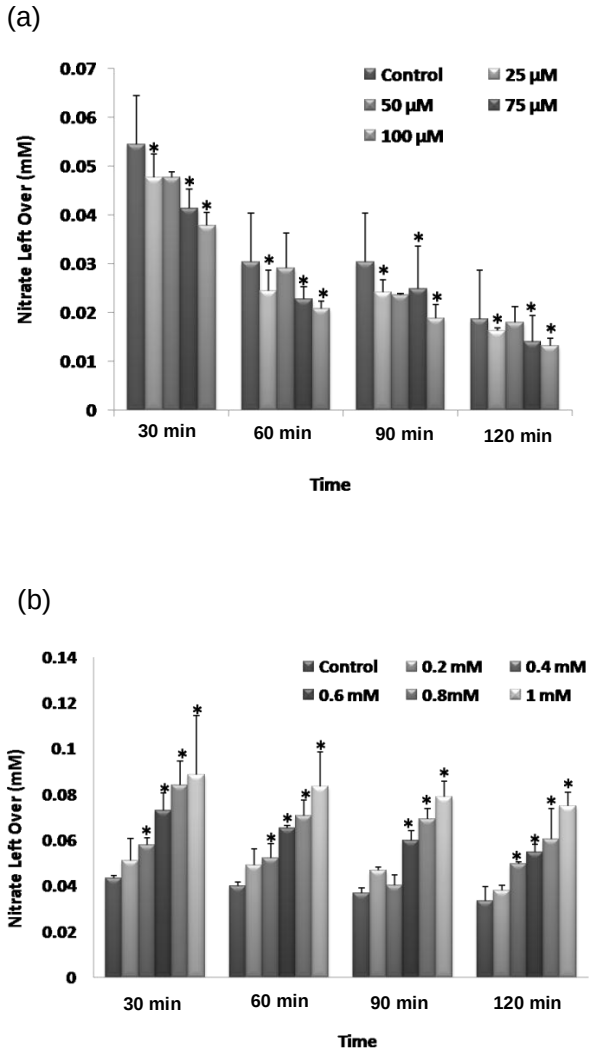

Figure 1. Effect of (a) Verapamil and (b) Na_3VO_4 on nitrate uptake. Data represents the average of nitrate left over (mM) in triplicates ± standard error. A (*) indicate statistical significance.

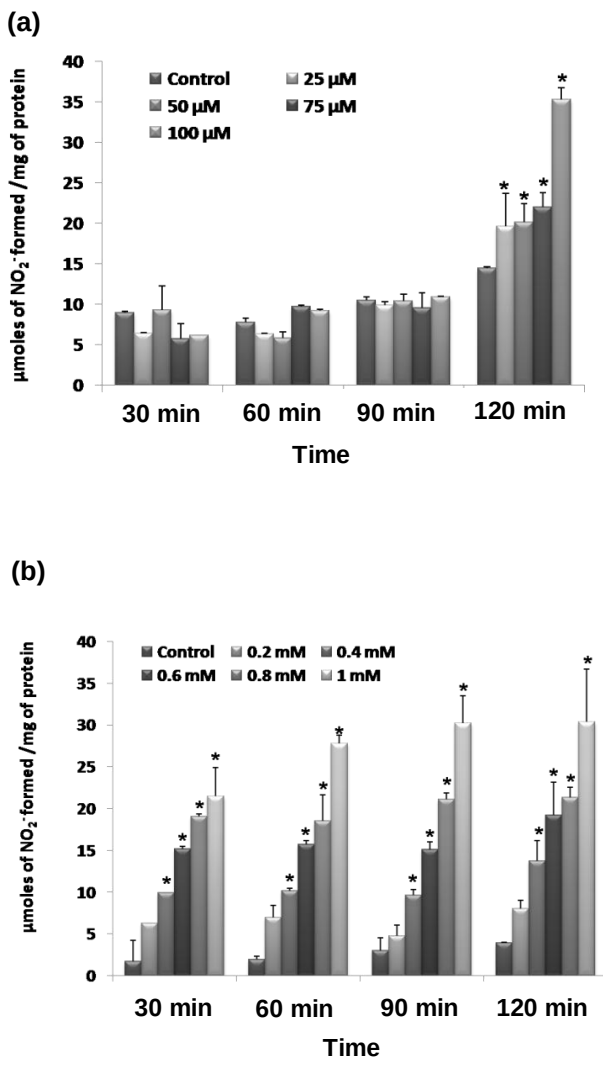

Figure 2. Effect of (a) Verapamil and (b) Na_3VO_4 on NR activity. Data represents the average of triplicates ± standard error. A (*) indicate statistical significance.

average of at least three replicates per treatment; means ± SE is shown in the figures. Statistical analyses were carried out using the GraphPad Prism software package (v. 4.02; GraphPad Prism Software Inc, San Diego, CA). Statistical significance between means was determined by using one-way ANOVA and the Tukey's multiple comparison tests at $p < 0.05$.

RESULTS

Effect of inhibitors on nitrate uptake

In order to study the role of Ca^{2+} ion channel, NU was examined in the presence of verapamil. Data presented in Figure 1a shows that NU increases in both control (NO_3^- repleted cells) as well as the inhibitor treated samples as indicated by the reduction of nitrate from the external medium. When compared with the control, NU was significantly higher at 25, 75 and 100 µM of verapamil ($p < 0.05$), in all the time points. Though not significant ($p > 0.05$), the rate of NU at 50 µM exhibited almost similar pattern as that of 25 µM of verapamil.

Concentration of Na_3VO_4 was chosen based on the available literature (McCartney et al., 1997). Data presented in Figure 1b shows that NU was inhibited in the presence of Na_3VO_4. The inhibition was proportional to the concentrations of Na_3VO_4 and was significant at the concentrations 0.4 to 1 mM Na_3VO_4 (Figure 1b) ($p < 0.05$).

Effect of inhibitors on nitrate reductase activity

Data presented in Figure 2 shows that NR activity increases in both controls as well as the treated samples as indicated by the formation of nitrite. In the presence of

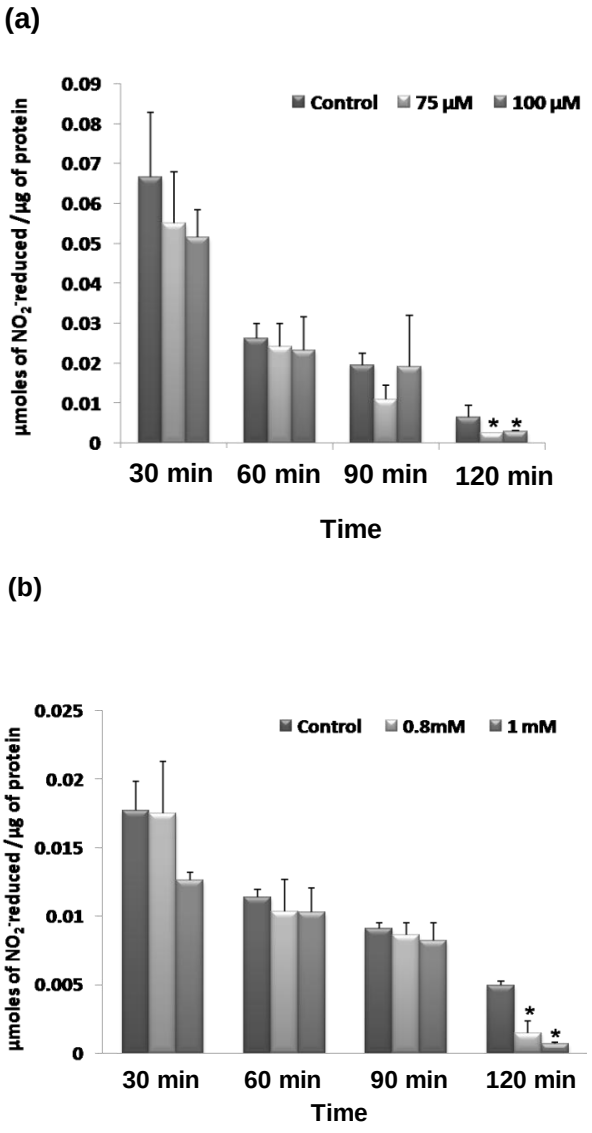

Figure 3. Nitrite reductase activity with (a) Verapamil and (b) Na_3VO_4. NiR activity was assayed as described in materials and methods. Data represents the average of triplicates ± standard error. A (*) indicate statistical significance.

verapamil, *S. platensis* showed a significant increase in NR activity (Figure 2a). In controls (without verapamil), NR activity was induced by addition of NO_3^-. This induction was significantly higher after 120 min in the filaments treated with verapamil. Verapamil at the concentration of 100 µM showed the highest NR activity. NR activity increased in the presence of Na_3VO_4 (Figure 2b) and was significant in concentrations between 0.4 and 1 mM ($p < 0.05$).

Effect of inhibitors on nitrite reductase activity

Like NR, NiR enzyme in the verapamil treated cells showed (Figure 3a) a fast rise in the activity as compared with the controls. After 120 min NiR activity was significantly higher in the presence of at 75 and 100 µM of verapamil ($p < 0.05$) (Figure 3a) when compared with that of the controls. These effects of verapamil have been observed despite the presence of optimal amount of calcium (40 mgL^{-1}) in the external medium.

In Na_3VO_4 treated cells, NiR activity showed an increase in the activity as compared with the controls (Figure 3b). After 120 min when compared with that of the controls, NiR activity was significantly higher in the presence of Na_3VO_4 at concentrations between 0.8 and 1 mM ($p < 0.05$) (Figure 3b). Thus, we conclude that NiR activity increases significantly in the presence of Na_3VO_4.

Effect of inhibitors on glutamine syhthetase activity

The data on Figure 4a represents the GS activity after 90 and 120 min of verapamil treatment. GS activity increases in both NO_3^- repleted control as well as the treated samples as indicated by the formation of γ-glutamylhydroxamate -iron (γGH-Fe) complex with time. However, verapamil treated cells showed an insignificant decrease in GS acvitiy ($p > 0.05$) (Figure 4a).

Figure 4b represents the GS activity after 90 and 120 min of Na_3VO_4 treatment. Like verapamil, GS activity increases in both NO_3^- repleted controls as well as Na_3VO_4 treated samples with time. However, when compared with that of the NO_3^- repleted controls (free of inhibitor), GS activity was significantly inhibited at 0.6 mM and 1mM of Na_3VO_4 ($p<0.05$) as indicated by the decrease in the formation of γGH-Fe complex (Figure 4b).

DISCUSSION

Though it has been proposed that Ca^{2+} may merely act as a chemical switch in signal transduction (Scrase-Field and Knight, 2003), it is assumed that the spatio-temporal components of the increase in Ca^{2+} signature dictates the outcome of the cellular end response (Ng and McAinsh, 2003). In heterocystous cyanobacteria, Ca^{2+} transient was triggered by the withdrawal of combined nitrogen from the external medium (Torrecilla et al., 2004). However, the extent of regulation involving Ca^{2+} ion channel in nitrate assimilatory pathway of non-nitrogen fixing *S. platensis* is not very clear.

Verapamil inhibits this Ca^{2+}-mediated response by holding the Ca^{2+} binding domain in an inactive conformation by an allosteric interaction with the gating mechanism associate with these domains (Ertel and Cohen, 1994; Triggle, 1982). Verapamil mainly gets access to the binding domain when the channel is open. As an organic cation it blocks the channel by interfering with Ca^{2+} ion binding to the extracellular mouth of the binding pore (Abernethy and Schwartz, 1999; Betkowski

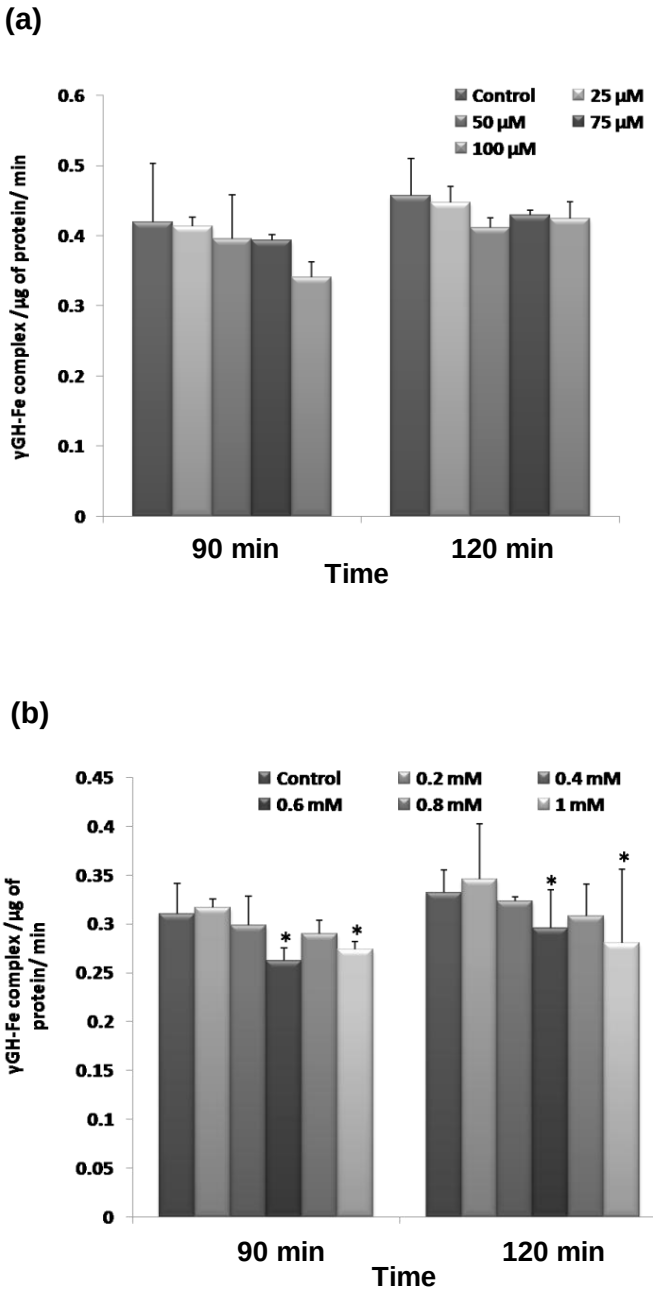

Figure 4. GS activity in the presence of (a) Verapamil and (b) Na_3VO_4.Data represents the average of triplicates ± standard error. A(*) indicate statistical significance.

and Hauptman, 2000). Recently genome sequencing of *S. platensis* have revealed the presence of extracellular Ca^{2+} binding domains, with a number higher than any other cyanobacteria (Fujisawa et al., 2010). Based on our results we suggest that one site of verapamil action might be these extracellular Ca^{2+} **-** binding domains.

We find that in *S. platensis*, despite the presence of Ca^{2+} in the medium, verapamil leads to a significant increase NU, NR and NiR activity. Singh and Singh (2000) have reported similar results in Ca^{2+} starved *S. platensis*. Ca^{2+} starvation lead to an increase in NR activity and decrease GS activity (Singh and Singh, 2000). From our results, it is clear that steady state NU, NR and NiR activities are dependent over Ca^{2+} influx into the cells from external medium and not just the presence of Ca^{2+} in the medium.

Furthermore, cytoplasmic concentration of Ca^{2+} has been related to changes in intracellular pH, although how protons and Ca interact is not known (Felle, 1988; Bush, 1995). In *Anabaena* it is reported that cellular Ca^{2+} level increases the intracellular pH. This ability of Ca^{2+} is correlated with an inhibition of net proton uptake by increasing Ca^{2+} influx from the external medium conditions (Giraldez-Ruiz et al., 1999). Nitrogen starvation leads to a decrease in intracellular pH (Karagiannis et al., 2001). Thus presences of verapamil have again lead to concentration dependent alteration in intracellular pH. It was also demonstrated that intracellular pH was potential regulators of the nitrate assimilatory enzymes acting upon it through a kinases mediated signaling system (Deng et al., 1991; Langendorfer et al., 1988). Thus, presence of verapamil has hindered Ca^{2+} influx, decreasing intracellular pH in concentration dependent manner, activating kinases. The activated kinases would then enhance NU, NR and NiR activity. However, GS activity showed an insignificant decrease in activity, which leads to a gesticulation that unlike NU, NR and NiR activity, GS activity might be under positive regulation of Ca^{2+} ions. However, the mechanism behind this regulation is yet to be studied.

Orthovanadate is a non specific inhibitor of Ca^{2+} transport and Ca^{2+}-stimulated ATPase as demonstrated by Lockau and Pfeffer (1983) in other cyanobacterium. The inhibition of ATPases and Ca^{2+} uptake leads to a decrease in cytoplasmic pH (Bagar et al., 2009; Giraldez-Ruiz et al., 1999). Thus, we conclude the concentration dependent increase in NR and NiR activities in the presence of orthovanadate are in agreement with that of our results with verapamil treatment. However, NU and GS activity are inhibited by orthovanadate. Unlike NR and NiR acitivity, nitrate uptake and GS activity in cyanobacteria are ATP dependent processes (Hu et al., 2000; Ali et al., 2008). Thus, we propose that inhibition of NU and GS activity with orthovanadate was due to the inhibition of ATPases by this inhibitor (Lockau and Pfeffer 1983).

In fungi, it is reported that vanadate at a concentration of 0.1 mM, inhibits the development of the NR activity leading to the accumulation of intracellular nitrate (Schloemer and Garrett, 1974). However, no such inhibition was seen in *S. platensis* even at higher concentration (1 mM) of vanadate. NR activity increases significantly in the presence of vanadate. Thus we presume that unlike fungi, cyanobacterial NR are resistant to vanadate inhibition.

We conclude that nitrate uptake and assimilation in *S. platensis* could be largely an intracellular pH related phenomenon. Steady state levels of NU, NR and NiR

activities are under the regulation of Ca^{2+} influx. Thus, present study gives an idea on possible mode of regulations operating the nitrate assimilatory pathway of *S. platensis*. The details on in-depth molecular events in the process are yet to be uncovered. Considering *S. platensis* as a food supplement, such a study on this indispensible organism would be the need of the hour.

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