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Requirement of Calcium for the Biosynthesis of Shinorine, a Mycosporine-Like Amino Acid (MAA) in *Anabaena variabilis* PCC 7937

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ABSTRACT

The role of calcium chloride in biosynthesis of ultraviolet (UV) -absorbing mycosporine-like amino acid (MAA), shinorine (λ_{max} 334nm) was studied under PAR+UVR irradiation in the cyanobacterium *Anabaena variabilis* PCC 7937. To find out whether calcium and/or chloride is important for shinorine biosynthesis, five BGA media with different cation and anion combinations were designed in a matrix to substitute calcium and chloride. Growth and shinorine induction studies clearly showed that calcium was required for shinorine biosynthesis as shinorine concentration increased when chloride was substituted but having calcium in the media. Contrary to this, shinorine synthesis was stopped after 48 h of irradiation and no further induction of shinorine was noticed in media with substituted cation for calcium but having chloride ion. This negative effect on shinorine biosynthesis was not correlated with the growth as no effect on growth was observed in the media substituted for calcium ion. The results of calcium channel blocker experiment further hinted at the requirement of calcium for shinorine biosynthesis and involvement of calcium channel in MAAs biosynthesis under UV radiation.

INTRODUCTION

In the past few years, mycosporine-like amino acids (MAAs) have received much attention for their putative role in UV photoprotection because of their strong absorption between 310 and 362nm and high molar extinction coefficients (ε = 28,100 - 50,000 M^{-1} Cm^{-1}). These compounds were originally implicated in UV-induced sporulation[1] in fungi. They are small (<400 Da), colourless, water-soluble compounds comprising of cyclohexenone or cyclohexenimine chromophore conjugated with the nitrogen substituent of an amino acid or its imino alcohol[2,3]. Generally, the ring system contains a glycine subunit at third carbon atom. Some MAAs also contain sulfate esters or glycosidic linkages through the imine substituents[4,5]. Differences among the absorption spectra of MAAs are due to the variation in the attached side groups and nitrogen substituents. There are more than 22 MAAs synthesized by various organisms, ranging from bacteria, cyanobacteria, macroalgae, phytoplankton but not in animals where they are accumulated through food chain[6].

There are several studies that report the effect of various abiotic stress factors such as UVR, salt, heat, desiccation and ammonium on the synthesis of MAAs in cyanobacteria as well as in other organisms[7,8]. However, there was no report for the effect of nutrient deficiency on MAAs biosynthesis. In the present investigation, the role of calcium chloride in the biosynthesis of MAA shinorine in a cyanobacterium *Anabaena variabilis* PCC 7937 was studied.

MATERIALS AND METHODS

Experimental organism and growth conditions

Anabaena variabilis PCC 7937 procured from Pasteur culture collection (Institute Pasteur, France), was grown in nitrogen free BGA medium[9] in a culture room at 20 ± 2°C and fluorescent light illumination of 12 ± 2 W m^{-2}. The BGA medium contained K_2HPO_4, 0.057 mM (0.01 g/L); $MgSO_4.7H_20$, 0.101 mM (0.025 g/L); Na_2CO_3, 0.18 mM (0.02 g/L); $CaCl_2.2H_2O$, 0.5 mM (0.0735 g/L); $Na_2SiO_3.5\ H_2O$, 0.207 mM (0.044 g/L); ferric citrate, 0.0133 mM (0.0035 g/L); citric acid, 0.018 mM (0.0035 g/L) in 1000 mL of distilled water. All experiments were conducted with exponentially growing cultures equivalent to 0.2 mg/mL dry weight.

Experimental setup

To investigate whether calcium and/or chloride is essential for MAAs biosynthesis, $CaCl_2$ in BGA medium was substituted with various cation and anion combinations. Different BGA media were designed in a matrix with $MgCl_2$ or $CaSO_4$ or $MgSO_4$ or without $CaCl_2$ at a same molar concentration of $CaCl_2$ in BGA medium to substitute the $CaCl_2$ while other components of the medium were same as mentioned above. The normal BGA medium with $CaCl_2$ was treated as a control and growth as well as MAA biosynthesis was studied in these media.

Effect of media composition on growth and MAA synthesis

The above mentioned five BGA growth media having $CaCl_2$ (control) or $MgCl_2$ or $CaSO_4$ or $MgSO_4$ or without $CaCl_2$, were prepared. For MAA induction experiments, 100 mL of cyanobacterial cell suspension were centrifuged using a JA 20 rotor (Beckman instruments, Palo Alto, CA, USA) at 500 × *g* for 5 min, washed three times with sterile double distilled water and subsequently transferred into the above mentioned five (three replicates for each medium) autoclaved media and allowed to grow for 6 days under above mentioned growth conditions. After 6 days, these cultures were used for the MAA induction experiments as described below. For estimation of the growth in these five media, cells were washed three times with sterile water and 1 mL of this suspension was used to inoculate 100 mL of these media (three replicates each). All cultures were transferred to the above-mentioned growth conditions and growth was estimated for consecutive 10 days by taking the O.D. at 665nm. The values obtained were plotted against time to obtain the growth curve in these media.

Shinorine induction experiment

For induction of the shinorine, cells grown in five media were exposed continuously for 72 h to artificial UV-B, UV-A (Ultraviolet-A; 315 - 400 nm), and PAR (Photosynthetically active radiation; 400 - 700 nm) in sterile glass Petri dishes (75 mm in diameter). 295nm cut-off filter foils (Ultraphan, Digefra, Munich, Germany) were placed over Petri dishes to achieve a PAR+UV-A+UV-B regime (Fig. 1A) as this radiation composition was found to induce the maximum concentration of the MAA shinorine in this cyanobacterium[18]. Visible (PAR) light was achieved by Osram L 36 W/32 Lumilux de luxe warm white and radium NL 36 W/26

Universal white tubes (Germany) while ultraviolet-B TL 40 W/12 fluorescence tubes (Philips, The Netherlands) were used as a source of UV-B irradiation. Since UV-B tubes used in this study were also emitting UV-A irradiation no additional source for UV-A irradiation was used. Figure 1B shows the spectral irradiance of the light sources used in shinorine induction experiment as measured with a double-monochromator spectroradiometer (OL 754, Optronic Laboratories, Orlando, FL, USA). The irradiances effectively received by the samples were 0.89 W/m^2 for UV-B, 1.06 W/m^2 for UV-A and 10 W/m^2 for PAR based on the transmission characteristics of the cut-off filters (Fig. 1A).

All cultures were hand shaken several times during exposure to avoid self-shading. After 24, 48 and 72 h of exposure, 2 mL (three replicates for each treatment) of samples were collected from each treatment and further processed for determining the shinorine concentration. Samples were also withdrawn from each treatment at the beginning of the exposure (0 h).

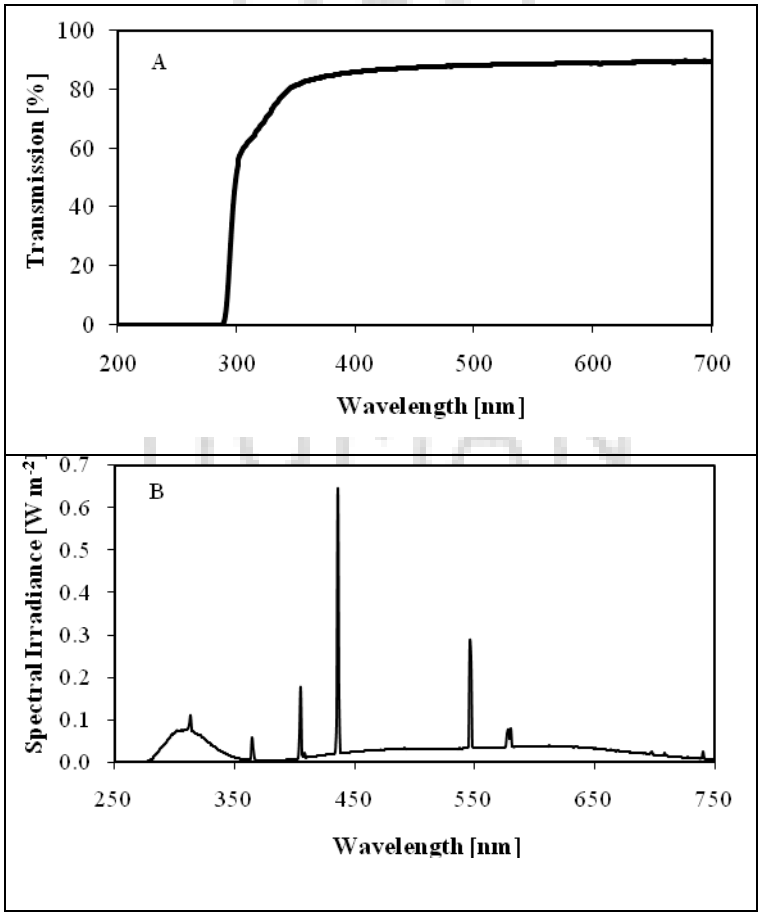

Figure 1. (A) Transmission spectrum of 295nm cut-off filter and (B) spectral characteristics of the light source used in shinorine induction experiments.

Effect of calcium channel blocker on shinorine synthesis

The effect of calcium channel blocker nifedipine (Sigma-Aldrich, Steinheim, Germany) on shinorine synthesis was studied to support our results obtained from the media composition experiment. Nifedipine is a dihydropyridine calcium channel blocker which blocks the L-type calcium channels. Cyanobacterial cultures in normal BGA medium[9] were treated with 0, 50, 100 or 200 µM nifedipine dissolved in DMSO and exposed to above-mentioned radiation conditions for the induction of shinorine. After 72 h of exposure, samples were collected and further processed for the shinorine concentration determination. The maximum amount of DMSO used in nifedipine treatment was also added to the culture to see the effect of DMSO on shinorine biosynthesis if any. After 72 h of exposure cultures were also plated on solid medium and colonies were counted after two weeks to see the effect of added nifedipine on survival of the organism. In addition, the effective photosynthetic quantum yield (Y) was also measured using the portable pulse amplitude modulated fluorometer (PAM 2000, Walz, Effeltrich, Germany) in presence of different concentration of nifedipine after 72 h of exposure.

Shinorine extraction

After sampling, cells were harvested by centrifugation 5000 × g for 5 min (GP centrifuge, Beckman, Palo Alto, USA) and shinorine was extracted overnight at 4°C in 2 mL of 100% methanol (HPLC grade). Thereafter, the aliquots were centrifuged at 5000 × g for 10 min and supernatants were transferred to fresh Eppendorf tubes. The resultant methanolic extracts were evaporated to dryness at 30°C in a vacuum evaporator and redissolved in 500 µl of double distilled water. A few drops of chloroform were added to this solution and after centrifugation at 5000 × g for 10 min water phase was taken carefully into new Eppendorf tubes to get rid of the contaminant photosynthetic pigments.

Quantification by HPLC

The aqueous solution of shinorine was filtered through 0.2 µm pore-sized microcentrifuge filters (Mikro-Spin Zentrifugen filter, Roth, Karlsruhe, Germany) and subjected to HPLC system (Waters 996 photodiode array detector, USA; pump L-7100, Darmstadt, Germany) equipped with a Licrospher RP 18 column and guard (5 µm packing; 250×4 mm I.D.). Shinorine was

quantified from the peak area obtained in chromatograms as described earlier[10]. Samples (50 µl) were injected into the HPLC column through a Waters 717 plus autosampler (USA). The wavelength for detection was 330nm and mobile phase was 0.02% acetic acid (v/v in double distilled water) at a flow rate of 1 mL/min.

Statistical analysis

All experiments were performed three times with consistently the similar results and results are presented as mean values of three replicates Shinorine concentration under different treatment was analysed by one way ANOVA[11] and once significant difference detected post hoc multiple comparisons were made by using Tukey test ($\alpha = 0.05$).

RESULTS

This study was conducted with a view to find out whether calcium and/or chloride is required for biosynthesis of the shinorine in studied cyanobacterium. The results obtained from the growth experiment in different BGA media substituted for calcium or chloride revealed that growth was not inhibited in media having $MgCl_2$ or $CaSO_4$ or $MgSO_4$ or without $CaCl_2$ (Fig. 2). This indicated that there was no negative effect of the omission and/or substitution of calcium or chloride on the growth and *A. variabilis* PCC 7937 can grow in all growth media substituted for calcium or chloride. Once it became clear that omission and/or substitution of calcium and/or chloride form the growth medium do not have any negative effect on the growth of cyanobacterium, we further studied the shinorine synthesis in these media to find out whether calcium and/or chloride is required for its synthesis.

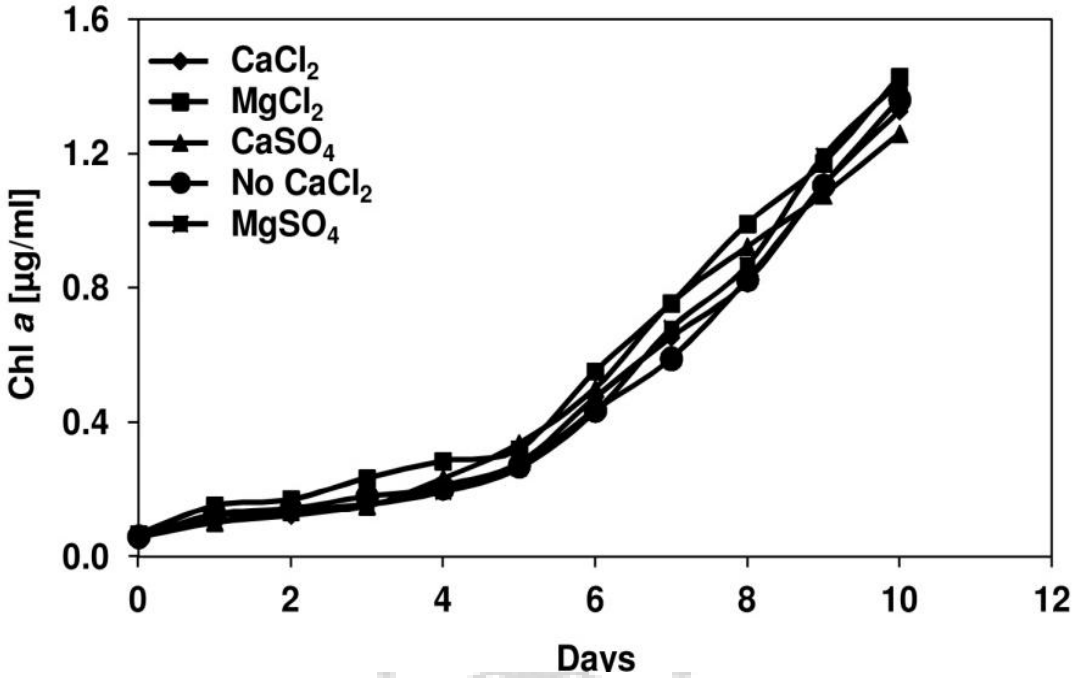

Figure 2. Growth of *Anabaena variabilis* PCC 7937 in different BGA media substituted for either calcium or chloride. Normal BGA medium with $CaCl_2$ acted as control. After inoculation cultures were transferred to normal growth conditions (see material and methods) and growth was monitored daily by taking the O.D. at 665nm.

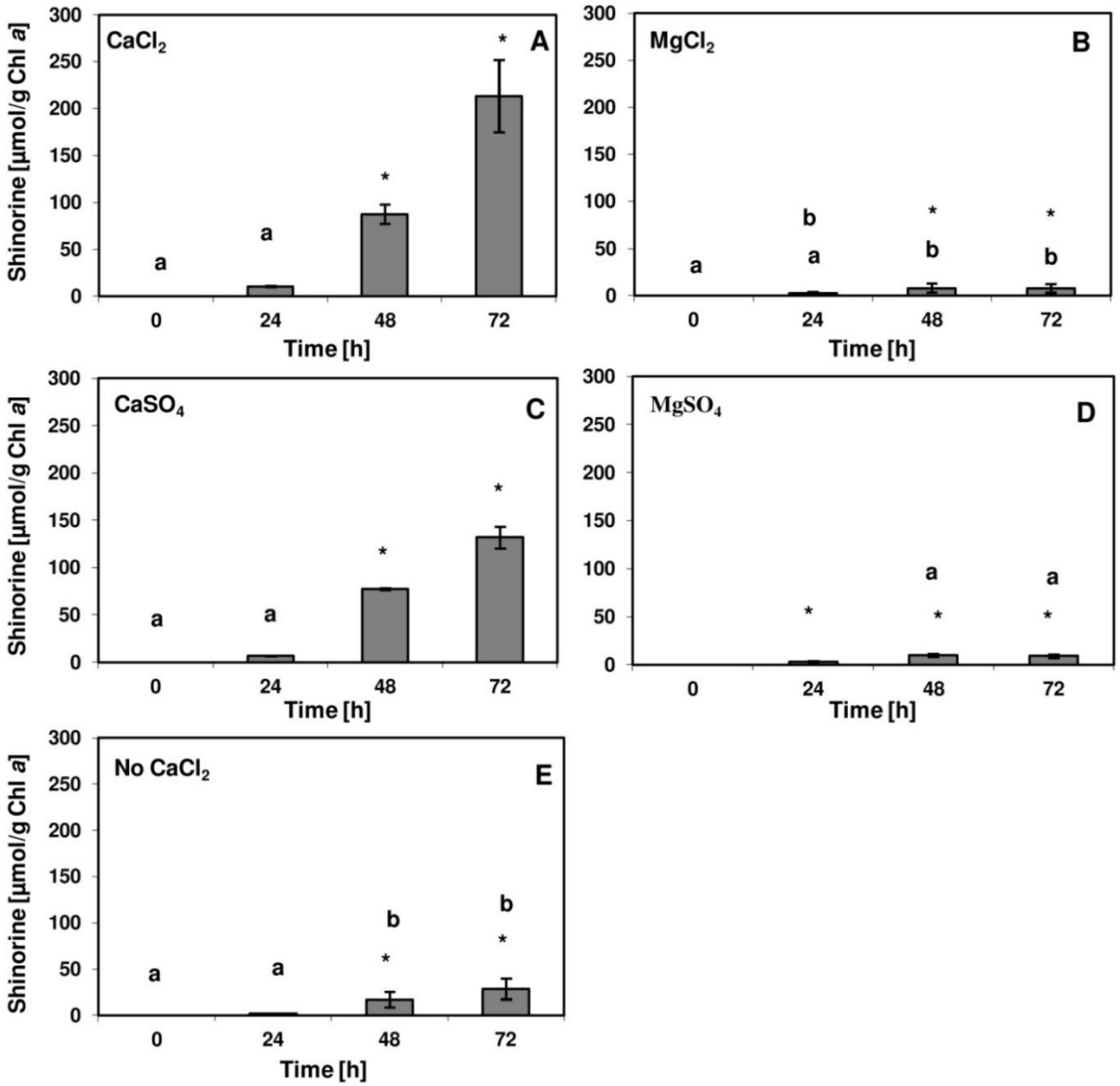

Figure 3. Concentration of shinorine (µmol/g Chl *a*; mean ± SD, n = 3) during 72 h of exposure to PAR+UVR radiation in different BGA media substituted for either calcium or chloride. Normal BGA medium with $CaCl_2$ acted as control. Similar letters over the bars indicate no significant difference ($P > 0.05$) between treatments, whereas the asterisks represent significant differences from 0 h ($*P < 0.05$).

The results from the HPLC analysis revealed that there was a significant increase in shinorine concentration at each time interval in normal BGA medium having $CaCl_2$ (control) except for 24 h where the shinorine concentration was not significantly different from the initial value (0 h) (Fig. 3A). There was no significant increase in shinorine concentration after 24 h of exposure in BGA medium lacking calcium but having chloride (with $MgCl_2$), from the initial value (0 h)

(Fig. 3B). The shinorine concentration was significantly higher after 48 and 72 h of exposure from the initial value; however, there was no significant difference between 24, 48 and 72-h exposed samples in same BGA medium lacking the calcium ion (Fig. 3B). There was no significant difference in shinorine concentration between 0 and 24-h exposed samples in BGA medium lacking chloride but having calcium ($CaSO_4$), however, after 48 and 72 h of exposure, the shinorine concentration was significantly higher from the initial value (0 h) in the same medium (Fig. 3C). There was also significant difference between 48 and 72-h exposed samples and shinorine concentration was highest after 72 h of exposure in same medium (Fig. 3C). In a medium lacking both calcium and chloride (with $MgSO_4$), the shinorine concentration was significantly higher after 24 h of exposure from the initial value (0 h) and there was no significant difference between 48 and 72-h exposed samples (Fig. 3D). Similar pattern was also obtained in a medium where both calcium and chloride ions were absent and no other cation and anion combination was added to compensate the deficiency of calcium chloride (Fig. 3E). There was a significant increase in shinorine concentration after the first 24 h of exposure from 0 h in a medium lacking both calcium and chloride ions. However, the shinorine concentration was not significantly different in 48 and 72-h exposed samples in same medium (Fig. 3E).

The results from the comparison of shinorine concentration in different media at each time interval revealed that the concentration of shinorine was always highest in a medium having calcium chloride followed by the one having calcium sulphate (Fig. 4). After 24 h of exposure, the shinorine concentration was significantly higher in medium having calcium chloride or calcium sulphate from the others, which were having substituted cation or anion for calcium or chloride of original BGA medium (Fig. 4A). There was no significant difference in shinorine concentration in medium having $MgCl_2$, $MgSO_4$ or no $CaCl_2$ after the same time of exposure (Fig. 4A). After 48 h of exposure, the shinorine concentration was similar in medium having $CaCl_2$ or $CaSO_4$, however, in other media (with $MgCl_2$, $MgSO_4$ or no $CaCl_2$) the shinorine concentration was significantly less from these two media (with $CaCl_2$ or $CaSO_4$). There was also no significant difference in shinorine concentration in media having $MgCl_2$, $MgSO_4$ or no $CaCl_2$ after the same time of exposure (Fig. 4B). Similar pattern was also observed after 72 h of exposure and shinorine concentration was highest in media having calcium (Fig. 4C). The shinorine concentration was highest in medium having $CaCl_2$ followed by one having $CaSO_4$, however, in other media (with $MgCl_2$, $MgSO_4$ or no $CaCl_2$), the shinorine concentration was

significantly lower from media having $CaCl_2$ or $CaSO_4$ (Fig. 4C). Thus, results clearly indicate that calcium is important for biosynthesis of the MAA in a cyanobacterium *A. variabilis* PCC 7937.

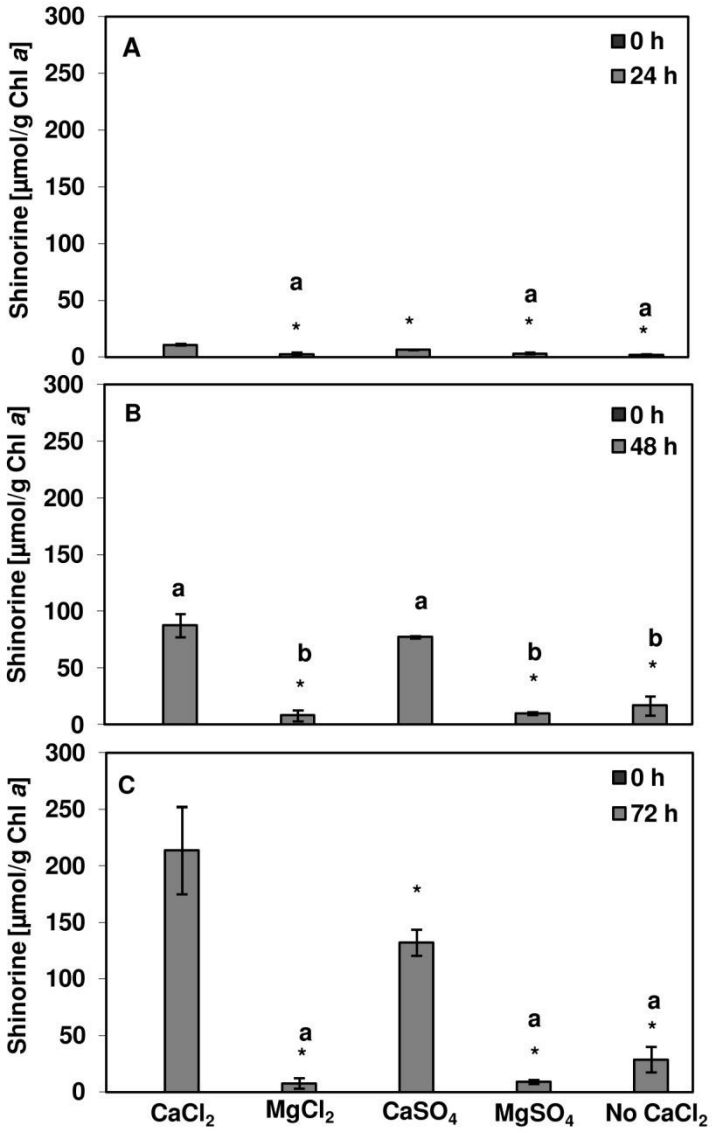

Figure 4. Comparison of shinorine concentration (µmol/g Chl *a*; mean ± SD, n = 3) at each time interval after exposure to PAR+UVR radiation in different BGA media substituted for either calcium or chloride. Normal BGA medium with $CaCl_2$ acted as control. Similar letters over the bars indicate no significant difference ($P > 0.05$) between treatments, whereas the asterisks represent significant differences from control ($*P < 0.05$).

In further study, the effect of L-type calcium channel blocker nifedipine was studied on the synthesis of shinorine (Fig. 5). The results obtained indicate that the synthesis of shinorine was

significantly inhibited in the presence of 200 µM nifedipine while there was no significant difference in 0, 50, 100 µM nifedipine or only DMSO-treated samples, indicating that the effect was not due to the DMSO. The inhibition of shinorine biosynthesis in the presence of 200 µM nifedipine indicates that the calcium and L-type calcium channels are required for MAAs biosynthesis. We also tested the survival of cyanobacterium after 72 h of exposure in the presence of different concentrations of nifedipine by counting the number of colonies appeared after two weeks. The results indicate that there was no negative effect of added nifedipine concentration on the survival of cyanobacterium as there was no significant difference in number of colonies appeared in 0, 50, 100 or 200 µM nifedipine treated samples (data not shown). The results from the PAM analysis also revealed that there was no inhibitory effect of added nifedipine on physiology of the cyanobacterium as after 72 h of exposure the photosynthetic yield was 0.24 ± 0.04, 0.37 ± 0.04, 0.35 ± 0.02 and 0.27 ± 0.01 in 0, 50, 100, and 200 µM nifedipine treated samples, respectively(data not shown).

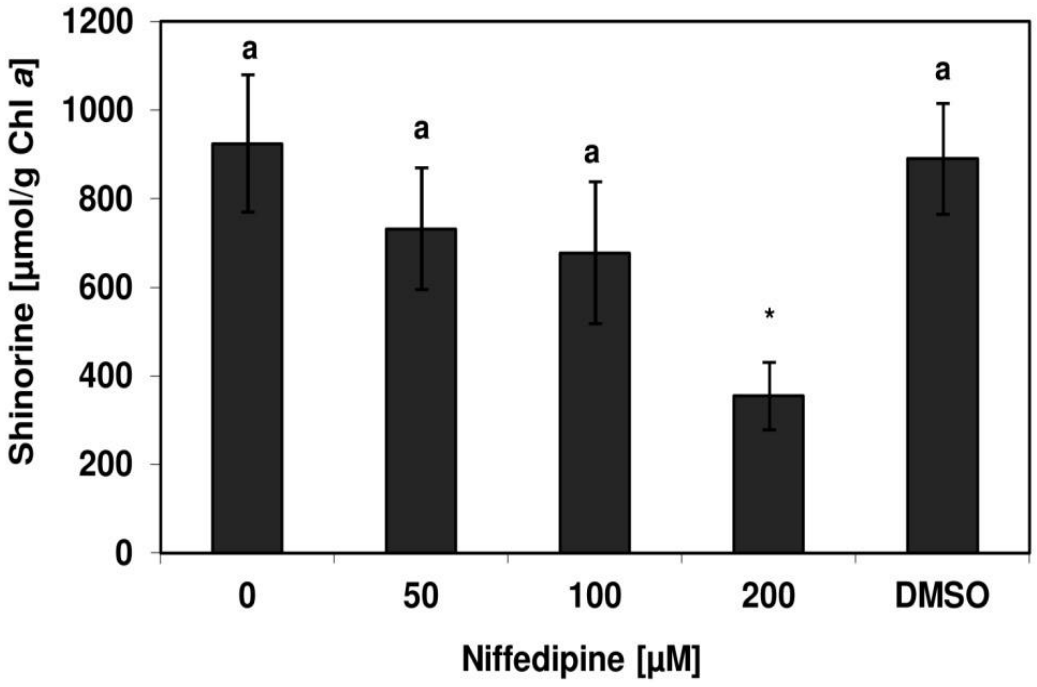

Figure 5. Concentration of shinorine (µmol/g Chl a; mean ± SD, n = 3) after 72 h of exposure to PAR+UVR radiation in presence of different concentrations of calcium channel blocker nifedipine. The effect of maximum amount of DCMU (solvent for nifedipine) used in experiment was also tested on shinorine biosynthesis. Similar letters over the bars indicate no significant difference (P > 0.05) between treatments, whereas the asterisks represent significant differences from 0 µM nifedipine treated samples (*P < 0.05).

DISCUSSION

The high-energetic UV-B has the greatest potential for cell damage caused by both the direct effect on DNA and proteins and the indirect effects *via* the production of reactive oxygen species[12-15]. It has been reported to affect the morphology[16] and several physiological as well as biochemical processes such as survival, growth, pigmentation, photosynthetic oxygen production, motility, N_2 metabolism, phycobiliprotein composition and $^{14}CO_2$ uptake in cyanobacteria[17-19]. As a mitigation strategy against lethal UV radiation, cyanobacteria have been reported to induce UV-absorbing MAAs[6,10] and there are several evidences that indicate the photoprotective role of MAAs in various organisms[20,21]. The photodegradation and photophysical characteristics study of MAAs have also shown that these compounds are capable of effectively dissipating absorbed radiation without producing reactive oxygen species[22].

Recently, we have found that bioconversion of shinorine into the secondary MAA is regulated by the sulfur deficiency in *A. variabilis* PCC 7937 (unpublished data). Thus, MAAs are not only important under UV, salt and ammonium stresses[7] but also have the role of nutrient deficiency. In the present study during our experiment, no inhibitory effect on the growth was found in different media substituted for either calcium or chloride and growth pattern was similar to the control (with $CaCl_2$) in all media (with $MgCl_2$ or $MgSO_4$ or no $CaCl_2$) (Fig. 2). However, in longer term the growth of cyanobacterium is most likely expected to be inhibited in the absence of $CaCl_2$. Growth of cyanobacterium in all these media can be explained by the fact that the water used for making the BGA medium was found to have traces of calcium chloride and thus supported the growth of cyanobacterium even in the absence of calcium chloride in growth medium. This suggested that the effect of ion substitution on shinorine synthesis was not correlated with the growth and results clearly indicated that calcium not chloride is required for the synthesis of MAA in this cyanobacterium, since synthesis of shinorine was inhibited only in media substituted for calcium and traces of calcium, if any present in medium were not enough for its biosynthesis. The possible involvement of calcium in MAA biosynthesis also gets support from the study of Richter et al.[23] in which these workers reported an increase in cytosolic calcium concentration after UV+PAR irradiation in a filamentous cyanobacterium *Anabaena* sp. These workers also reported the opening of calcium channels in response to UV radiation and in another study dihydropyridine-sensitive L-type calcium channels were reported in *Anabaena*

sp.[24] These two studies supported the role of calcium and calcium channels under UV radiation in cyanobacteria. The result from present study clearly shows the requirement of calcium and L-type calcium channel in MAAs biosynthesis. The inhibitory effect of nifedipine on shinorine biosynthesis was true effect of inhibition of calcium influx as there was no effect observed on survival and photosynthetic yield of cyanobacterium in presence of used concentrations of nifedipine. Calcium plays an important role in cell and change in cellular calcium homeostasis regulates various cellular processes in both prokaryotes and eukaryotes[25,26]. Calcium regulates several cellular functions such as ionic balance, motility, gene expression, carbohydrate metabolism, mitosis, secretion and any stimulus that initiates a complex cellular response is supposed to involve calcium at some step in the cellular signal transduction pathway[25]. There are several studies that indicate involvement of the calcium in perception of signals in cyanobacteria[27-30], and calmodulin-like protein; an important component of calcium-dependent signaling has been also characterized in a cyanobacterium *Nostoc* sp. PCC 6720[31]. Thus, it is clear that calcium also plays an important role in signaling in the cyanobacteria.

Portwich and Garcia-Pichel[32] have reported that MAA synthesis can be induced by salt stress without PAR or UVR in a cyanobacterium *Chlorogloeopsis* strain PCC 6912 and distinguished between salt-dependent biochemical and light-dependent photosensory induction of MAA synthesis. These workers have also proposed a reduced pterin as putative UV-B specific photoreceptor chromophore for the light-dependent induction of the MAA shinorine. The prediction of a reduced pterin as UV-B specific photoreceptor chromophore for the induction of MAAs was based on an action spectrum obtained for MAAs induction and inhibition of MAAs synthesis in presence of an inhibitor of pterin biosynthesis and antagonist of excited states of pterin. However, it is important to mention here that these workers used only four cut-off filters at 295, 305, 320 and 335nm and that they have not ruled out the possibility of a second peak at shorter UV-B wavelengths. Similarly, Sinha et al[33] conducted a study to explicate the possible photoreceptor using inhibitors of the shikimate pathway, photosynthesis, protein synthesis and pterin synthesis as well as a quencher of the excited states of flavins and pterins. From their results, it was very difficult to confirm that pterin is the only photoreceptor for MAA induction since the MAA content decreased in presence of all inhibitors in comparison to the cells treated with UV-B only. Thus, the presence of a common signal pathway involving calcium for the induction of MAAs rather than specific one cannot be ruled-out and need more experiments to

see the role of calcium in signal perception for MAAs induction, if any. This is also supported by the fact that shinorine in *A. variabilis* PCC 7937 can be also induced by salinity and ammonium stresses in addition to UV stress[7]. Salt stress has been also reported to trigger transient increases in intracellular free Ca^{2+} concentration in cells of the nitrogen-fixing filamentous cyanobacterium *Anabaena* sp. PCC7120, suggesting cell sense this stress via Ca^{2+} signaling[29].

In conclusion, present study revealed that calcium is required for the biosynthesis of shinorine in a cyanobacterium *A. variabilis* PCC 7937 since biosynthesis of shinorine was inhibited in the absence of a calcium ion in the growth medium, irrespective of the growth. Thus, this study put forward a question about the involvement of calcium in shinorine biosynthesis and more experiments are needed to get more proof for calcium involvement in MAA synthesis and also to find out whether it is required for any chemical reaction involved in MAA synthesis or it acts as a signal molecule?

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