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ORIGINAL ARTICLE

Response of a rice-field cyanobacterium *Anabaena* sp. HKAR-7 upon exposure to ultraviolet-B radiation and ammonium chloride

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Abstract

Interactive effects of ultraviolet radiation (UVR), photosynthetically active radiation (PAR) and exogenously supplied ammonium chloride (NH_4Cl) was studied in the rice-field cyanobacterium *Anabaena* sp. HKAR-7. The cyanobacterium was cultured under varying NH_4Cl concentrations i.e., 0, 50, 200, 500, 1000 and 5000 µM and 200 µM (concentration) was found to be optimum for the growth of the cyanobacterium. Detrimental effects of UV-B exposure were observed on photosynthetic pigments such as chlorophyll *a* (Chl *a*), carotenoids and phycocyanin (PC). However, damage to these pigments was less in the cyanobacterial samples supplemented with NH_4Cl. Contents of Chl *a* and PC in cyanobacterial cells decreased upon UV-B exposure but decrement was less in the samples supplemented with NH_4Cl. Upon UV-B exposure, carotenoids content enhanced initially (till 15 days) during the course of treatment (21 days) but significant decrease (in carotenoids content) was observed in later phase of the experiment. From the results of photosynthetic activity, maximum quantum efficiency of PSII (*Fv/Fm*) and maximum electron transport rate (ETR_{max}), it could be concluded that exogenous supplementation of NH_4Cl (optimum concentration) helped in protecting the cyanobacterial cells from highly energetic UVR to certain extent. Another interesting observation was significantly higher levels of biosynthesis and accumulation of mycosporine-like amino acids (MAAs) in the cyanobacterial cells supplemented with NH_4Cl in comparison to non-supplemented cells. The purified MAA was identified to be phorphyra-334 as evidenced by UV/VIS absorption spectra, high performance liquid chromatography (HPLC) and electrospray ionization-mass spectrometry (ESI–MS).

Keywords Ammonium chloride · Cyanobacteria *Anabaena* · Mycosporine-like amino acids · Photoprotection · Ultraviolet radiation

Introduction

Ultraviolet radiation (UVR), the comparatively low waveband radiation, is composed of highly energetic photons which reach the Earth's surface along with solar radiation. In current scenario, UVR influx has increased on the Earth due to anthropogenically released ozone depleting compounds (Häder et al. 2015). Although, UV-B constitute less than 1% of the total incoming solar radiation (Vincent and Roy 1993), it severely affects crucial biomolecules such as DNA, RNA and proteins which are important for biochemical, physiological and genetic functioning of the cell (Sinha and Häder 2016; Rajneesh et al. 2019). Besides, in cyanobacteria, detrimental effects of UVR on pigmentation, phycobiliprotein composition, motility, N_2 metabolism, DNA, protein profile and $^{14}CO_2$ uptake have been well documented (Kannaujiya and Sinha 2015; Sinha

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and Häder 2016; Rajneesh et al. 2019). Enhanced production of reactive oxygen species (ROS) due to UVR leads to the destruction of D1 protein and photosystem (PS) II reaction centre and also disrupts photon absorption and electron transport (Xia et al. 2004). Decrease in photosynthetic quantum yields (*Fv/Fm*) has been observed in response to UVR in *Fischerella* sp. (Singh et al. 2017). Exposure to UVR results in breakage of the filaments (Qin et al. 2012) and inhibition of enzyme nitrogenase in N_2-fixing cyanobacteria leading to decreased nitrogen uptake (Kumar et al. 2003; Pandey et al. 2020). However, with due course of evolution, these photoautotrophs have developed several protective strategies for overcoming the harmful effects of lethal UVR (Pathak et al. 2019a) which ranges from behavioral to molecular levels. Accumulation and biosynthesis of UV screening compounds such as mycosporine-like amino acids (MAAs) is one such protective mechanism adopted by cyanobacteria to survive and sustain under such abiotic stresses (Richa 2015). Different studies have correlated the accumulation and biosynthesis of MAAs in cyanobacteria and algae in response to UV exposure (Lesser et al. 1996; Karsten et al. 1998a; Hoyer et al. 2001; Rastogi and Incharoensakdi 2013; Richa 2015). MAAs are water-soluble, low molecular weight nitrogenous compounds having high molar extinction coefficients ($\varepsilon = 28{,}100–50{,}000\ M^{-1}\ cm^{-1}$) with absorption band ranging in between 310–362 nm (Richa 2015; Ahmed et al. 2019; Singh et al. 2020). Apart from synthesis of UV-screening compounds, other repair mechanisms against UVR exposure involve synthesis of several enzymes and protein cofactors (Roy 2000) and nitrogen limitation results in less efficient repair mechanisms, hence making the light driven process of photosynthesis more sensitive to UVR (Litchman et al. 2002). Korbee-Peinado et al. (2004) found that biosynthesis of MAAs was stimulated in response to external nitrogen supplementation in form of ammonium in red alga *Porphyra columbina*. However, there is wide controversy regarding the factors regulating the accumulation and induction of MAAs and information regarding the effect of nitrogen supplementation in form of ammonium chloride (NH_4Cl) on biosynthesis and accumulation of MAAs in cyanobacteria are scarce (Banaszak and Neale 2001; Litchman et al. 2002). Therefore, the present investigation aims at studying the effects of UV-B and photosynthetically active radiation (PAR) on chlorophyll (Chl *a*), biliproteins, photosynthetic performance and biosynthesis of MAAs in *Anabaena* sp. HKAR-7, with and without the supplementation of optimum dose of exogenous nitrogen source in the form of NH_4Cl. Such study would help in understanding the photoprotective mechanisms that allow phototrophic organisms such as cyanobacteria to sustain and reproduce in brightly lit and nutrient rich habitats.

Materials and methods

Experimental setup

The cyanobacterium, *Anabaena* sp. HKAR-7, isolated and purified from the rice-fields of Banaras Hindu University, Varanasi, India, was selected for the present study. Microscopic analysis was done using light (CX21i, Olympus Corporation, Tokyo, Japan) and scanning electron microscope (EVO18 research, Zeiss, UK) (Supplementary Fig. 1). Morphological identification was done through monographs and standard taxonomic keys (Desikachary 1959), and molecular characterization was done by *16S rRNA* gene amplification and maximum likelihood method was utilized for phylogenetic tree mapping. Alignment of the sequence of *16S rRNA* gene fragment against known sequences present in the GenBank database was done using the BLAST program of NCBI search (Altschul et al. 1990). CLUSTALW was used for producing multiple alignments. The *16S rRNA* gene sequence of the cyanobacterium was classified into phylogenetic group as proposed by Desikachary (1959) to determine the genetic variability between and within the groups. A phylogenetic tree was constructed using the neighbor-joining algorithm (Saitou and Nei 1987) provided in MEGA 7 software (Kumar et al. 2016).

Autoclaved BG-11 (without nitrogen sources) medium was used for routine growth of the cyanobacterial cultures (Rippka et al. 1979) under axenic conditions at a temperature of 28 ± 2 °C, under continuous fluorescent white light ($12\ Wm^{-2}$). Cyanobacterial cultures were shaken manually four times a day in order to avoid clumping and shelf shading. Different concentrations of exogenous NH_4Cl (0:Control, 50, 200, 500, 1000 and 5000 µM) were used in nutrient medium for screening purpose and 200 µM concentration of NH_4Cl was found to be optimum for the growth of the cyanobacterium and hence was selected as optimum dose for further experiments. The homogeneous cultures of cyanobacteria (250 mL of culture with OD_{750} nm $= 0.68 \pm 0.2$; Path length 1 cm) were taken in sterile glass Petri dishes (120 mm in diameter) and were treated with artificial UV-B radiation and PAR in a UV chamber with and without exogenous supplementation of 200 µM NH_4Cl (HI Media, RM 717). The experiments were conducted under a 14:10 light/dark cycles with light intensity of 40 µmol photons $m^{-2}\ s^{-1}$ at 25 °C. Cyanobacterial samples containing Petri dishes were placed under UV-B TL 40 W/12 fluorescence tubes (TL20 W/01RS, Philips, Germany) and UV-B intensity of ~ 1 W m^{-2} for 4 h/day (d) from 11:00 to 15:00 was maintained by adjusting the distance of Petri dishes from the UV-B tube in the chamber. Each experiment was performed in triplicates. Cut-off

filter foils of 295 nm (Ultraphan; Digefra, Munich, Germany) were placed over each Petri dish for avoiding any exposure of UV-C radiation.

Measurement of photosynthetic pigments

Extraction of Chl *a* pigment was done by incubating the harvested cyanobacterial samples in 100% methanol for overnight at 4 °C in dark and quantification was done as per method given by Porra (2002), utilizing the absorbance values at 665.2 and 652 nm. Carotenoids were determined by the protocol described by Jensen (1978) with slight modification. Briefly, homogenized culture suspension was centrifuged at 10,000*g* for 10 min and supernatant was discarded. Pellet was dissolved in 85% acetone and calculation of carotenoids was done by recording the absorbance at 450 nm. For measurement of PC, cyanobacterial sample (10 mL) was centrifuged at 8000*g* for 15 min and dissolved the pellet in lysis buffer (pH = 8; 3 mL) followed by addition of 1 mg lysozyme in it. Samples were sonicated for 3–5 min and kept for overnight incubation at 4 °C followed by re-centrifugation at 10,000*g* for 5 min. Absorption spectra were recorded in the absorbance range of 200–700 nm against lysis buffer as a blank by using UV/VIS spectrophotometer (Hitachi 2900, Japan). The cellular PC content was calculated using equations described by Bryant et al. (1979).

Maximum quantum efficiency of PSII (*Fv/Fm*) and maximum electron transport rate (ETR_{max})

Pulse-amplitude-modulation (PAM) fluorometer (PAM-2500, Heinz Walz GmbH, 2008, Effeltrich, Germany) was used for determination of *Fv/Fm* values. Treated cyanobacterial samples were dark-adapted for 30 min in order to complete the process of oxidation of PSII reaction centres and the maximum (Fm) and minimum (F_0) fluorescent yields of PSII was observed in the dark-adapted state. The yields of Fm and F_0 were used for calculating the *Fv/Fm* values as per the formula given by Schreiber (2004). The photosynthetic electron transport rate (ETR) was calculated as per the formula:

$$\mathrm{ETR} = \left(\mathrm{Fm}' - \mathrm{Ft}\right)/\mathrm{Fm}' \times 0.84 \times 0.5 \times \mathrm{PPFD}$$

Fm′ = maximum fluorescence in light, Ft = steady state fluorescence in light, PPFD = photosynthetic photon flux densities.

Estimation of ETR was done from the operational PSII photochemical yield measured at different PPFD.

Extraction, partial purification and characterization of MAAs

For extraction of MAAs, cyanobacterial cells were harvested by centrifugation (Mikro 220R, Hettich, Germany) and pellets were resuspended in 100% methanol (HPLC grade), incubated overnight under dark conditions at 4 °C followed by homogenization. The aliquots were then centrifuged (5000*g*, 5 min) and supernatants were transferred to new microtubes and subjected to spectroscopic analysis between 250–700 nm using a UV–VIS spectrophotometer (U-2910, 2J1-0012, Hitachi, Tokyo, Japan). Analysis of the raw spectra (peaks) was done using UV Probe version software (Shimadzu Corp., Kyoto, Japan). The obtained supernatant (methanolic extracts) was evaporated at 40 °C in a vacuum evaporator (SPD111V, Thermo Electron Corp.) after spectroscopic analysis. The remaining residue was re-dissolved in 600 µL ultra-pure water. Chloroform (75 µL) was added to this solution followed by gentle vortexing and centrifugation (5000*g*, 5 min). After centrifugation, the water phase (uppermost) was transferred into fresh Eppendorf tubes to remove contamination by photosynthetic pigments (lipophilic) from the MAA (water-soluble). Finally, the samples were filtered by sterilized 0.2 µm pore size syringe filters (Axiva Sichem Biotech., New Delhi) and subjected to the high performance liquid chromatography (HPLC) analysis (Rastogi et al. 2012; Richa 2015).

HPLC analysis of UV-absorbing compound

Partially purified MAA was analyzed using HPLC (Waters, Elstree, UK), using a reverse phase semi-preparative column (symmetry prep C18, 7 µm particle size, 7.8 mm × 300 mm long) connected to an asymmetry guard column equipped with a Waters Photodiode array (PDA) detector. Samples (50 µL) were injected into the HPLC column and run at a flow rate of 1.0 mL min^{-1} using a mobile phase of 0.02% (v/v) acetic acid in ultra-pure water (Rastogi et al. 2012). The detection wavelength was 330 nm and the PDA scan wavelength was from 250–450 nm. The sharp peak, with a retention time (RT) of approximately 3.12 min was eluted and collected with the help of a fraction collector attached to the HPLC unit and quantification of MAA was performed by using the peak area (Richa 2015). Identification of the MAA was done by comparing the RT and absorption spectra.

Electrospray ionization-mass spectrometry (ESI–MS)

The HPLC purified fraction of MAA from *Anabaena* sp. HKAR-7 was subjected to ESI–MS to produce protonated molecules. Mass spectrum was recorded on an Amazon SL mass spectrometer (Bruker Daltonics Inc., Billerica, MA, USA). Cone voltage of 30 V was found to induce the formation of $(M+H)^{1+}$ with a mass range of 100–1000 *m/z*. Data was analyzed using the software Data Analysis 4.0 (Bruker Daltonics Inc., Billerica, MA, USA).

Statistics

All the experiments were conducted with three replicates to evaluate the means and standard deviation (mean ± SD). For evaluating the significance of the data, one-way analysis of variance was used. The significant data was used to determine post hoc multiple comparisons by using the Tukey test at the significance level of 0.05.

Results

On the basis of microscopic analysis, morphological identification, molecular characterization and phylogenetic tree mapping, the cyanobacterium was confirmed to be *Anabaena* sp. It is a member of order Nostocales, family Nostocaceae and is a filamentous and heterocystous cyanobacterium. The *16S rRNA* gene sequence of the cyanobacterium was submitted in NCBI database with an accession number KF857228. The phylogenetic tree revealed that the nearest homologues of *Anabaena* sp. HKAR-7 are *Anabaena constricta* MACC-177 (accession number MH702209) and *Nostoc muscorum* CCAP1453/8 (accession number HF678508) (Supplementary Fig. 2).

Interactive effects of UV-B radiation and ammonium (NH_4Cl) on *Anabaena* sp. HKAR-7

Changes in Chl *a* content were utilized for estimating the growth of *Anabaena* sp. HKAR-7 for six concentrations of NH_4Cl (0: Control, 50, 200, 500, 1000 and 5000 µM). We observed that 50, 200 and 500 µM concentrations of NH_4Cl positively influenced Chl *a* content. However, higher doses of NH_4Cl concentrations (1000 and 5000 µM) became toxic to the cyanobacterium. Chl *a* content increased gradually from initial value (0.20 µg mL^{-1}) to a maximum value of 2.80 µg mL^{-1} in 200 µM NH_4Cl followed by 50 µM NH_4Cl (2.60 µg mL^{-1}) treated samples at 21 days of experiment (Table 1).

Photosynthetic pigments and phycocyanin

Exogenous supplementation of NH_4Cl aided in maintaining higher levels of Chl *a* in the cyanobacterial cells exposed to PAR and PAR + UV-B as compared to non-supplemented samples till 15 days of treatment (Table 2). However, in cyanobacterial samples exposed to PAR + UV-B along with externally supplied NH_4Cl, Chl *a* content decreased after 21 days of treatment. Maximum Chl *a* content was observed in samples exposed to PAR + NH_4Cl (1.7 folds) for

Table 1 Effect of different concentrations of NH_4Cl (0, 50, 200, 500, 1000 and 5000 µM) on Chl *a* content in *Anabaena* sp. HKAR-7. Results are expressed as means of three replicates ± SD

Time (day)	Chl *a* (µg mL^{-1}) (mean ± SD)					
	0 µM NH_4Cl	50 µM NH_4Cl	200 µM NH_4Cl	500 µM NH_4Cl	1000 µM NH_4Cl	5000 µM NH_4Cl
0	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
3	0.37 ± 0.02	0.50 ± 0.05	0.50 ± 0.07	0.46 ± 0.01	0.48 ± 0.032	0.50 ± 0.11
6	0.83 ± 0.07	1.00 ± 0.05	1.00 ± 0.06	1.00 ± 0.04	0.96 ± 0.04	0.70 ± 0.09
9	1.20 ± 0.13	1.30 ± 0.12	1.70 ± 0.18	1.50 ± 0.05	1.50 ± 0.10	0.60 ± 0.10
12	1.35 ± 0.13	2.00 ± 0.05	2.10 ± 0.12	1.60 ± 0.06	1.50 ± 0.04	1.00 ± 0.12
15	1.83 ± 0.15	1.85 ± 0.14	2.10 ± 0.14	1.60 ± 0.01	1.50 ± 0.13	1.10 ± 0.09
18	1.90 ± 0.25	2.20 ± 0.16	2.50 ± 0.84	1.70 ± 0.02	1.80 ± 0.15	0.80 ± 0.13
21	2.03 ± 0.09	2.65 ± 0.135	2.80 ± 0.20	1.86 ± 0.01	1.67 ± 0.18	0.70 ± 0.11

Table 2 Effect of PAR, UV-B radiation and NH_4Cl (200 µM) on Chl *a* content in *Anabaena* sp. HKAR-7

Treatments	Chl*a* (µg mL^{-1}) (mean ± SD)		
	Time (days)		
	6	15	21
Control	$1.36 \pm (0.01)^{NS}$		
PAR	$1.80 \pm (0.14)^{a}$	$2.00 \pm (0.16)^{ab}$	$1.44 \pm (0.05)^{b}$
PAR + NH_4Cl	$1.99 \pm (0.13)^{a}$	$2.40 \pm (0.12)^{a}$	$1.46 \pm (0.09)^{b}$
PAR + UV-B	$1.52 \pm (0.12)^{a}$	$1.32 \pm (0.09)^{b}$	$0.90 \pm (0.08)^{c}$
PAR + UV-B + NH_4Cl	$1.76 \pm (0.17)^{a}$	$1.52 \pm (0.13)^{b}$	$1.20 \pm (0.08)^{c}$

Results are expressed as means of three replicates. Similar letters represent homogeneous mean group ($P > 0.05$)

15 days and least in PAR + UV-B treated samples (without NH_4Cl) (Table 2). It was observed that carotenoids content enhanced significantly in all the treated samples from initial value (25 µg mL^{-1}) after 6 days of exposure, followed by a decrease in later phase of treatment. This increment in the content of carotenoids was high in PAR + UV-B + NH_4Cl treated cyanobacterial samples (1.8 folds) as compared to PAR + UV-B (1.7 folds) treatment at 6 days. However, carotenoids content declined to about 1.9 and 1.3 folds in PAR + UV-B and PAR + UV-B + NH_4Cl treated samples respectively, after 21 days of exposure (Table 3). Initial PC content in the cells of *Anabaena* sp. HKAR-7 was recorded to be 0.364 mg mL^{-1}. Exposure of UV-B caused detrimental effects on PC content. However, this effect was quite less in the samples exposed to UV-B with exogenous NH_4Cl supplementation. Maximum decrease in the PC content was recorded in PAR + UV-B exposed samples (without NH_4Cl) at 21 days of treatment (Table 4).

Maximum quantum efficiency of PSII (*Fv/Fm*) and maximum electron transport rate (ETR_{max})

In order to assess the effects of given stress on tested cyanobacterium in terms of quantum efficiency of PSII (*Fv/Fm*) and ETR_{max}, we used PAM fluoremetre 2500. A strong correlation between values of *Fv/Fm* and healthiness of cyanobacterial samples was observed. For instance, control showed *Fv/Fm* value of 0.303. *Anabaena* sp. HKAR-7 maintained a relatively constant value of quantum yield, which increased slightly by 1.3 folds (0.3867 at 12 days) and one-fold (0.3037 at 9 days) in PAR + NH_4Cl and PAR treated samples respectively. The value of the *Fv/Fm* declined gradually in the samples exposed to PAR + UV-B (7.5 folds) and PAR + UV-B + NH_4Cl (5 folds) and remained constant till 21 days of exposure (Fig. 1a). Values of ETR_{max} showed similar trend as observed in *Fv/Fm* (Fig. 1b) and were found to be comparatively high in samples exposed to PAR + NH_4Cl as compared to PAR treatment. Exposure of UV-B without NH_4Cl resulted in most pronounced detrimental effect on photosynthetic activity of the cyanobacterium.

Table 3 Effect of PAR, UV-B radiation and NH_4Cl (200 µM) on carotenoids content in *Anabaena* sp. HKAR-7

Treatments	Carotenoids (µg mL^{-1}) (mean ± SD)		
	Time (days)		
	6	15	21
Control	$25 \pm (0.49)^{NS}$		
PAR	$30 \pm (2.30)^{a}$	$30 \pm (1.20)^{a}$	$23 \pm (4.60)^{a}$
PAR + NH_4Cl	$37 \pm (1.80)^{a}$	$35 \pm (4.00)^{a}$	$26 \pm (2.40)^{b}$
PAR + UV-B	$43 \pm (3.40)^{a}$	$26 \pm (2.90)^{b}$	$13 \pm (2.70)^{c}$
PAR + UV-B + NH_4Cl	$45 \pm (3.80)^{a}$	$32 \pm (3.80)^{b}$	$19 \pm (3.00)^{c}$

Results are expressed as means of three replicates. Similar letters represent homogeneous mean group ($P > 0.05$)

Effect of UV-B radiation and NH_4Cl on MAAs biosynthesis, partial purification and characterization

Absorption spectrum (UV/VIS) and HPLC analyses revealed significantly high induction of MAA phorphyra-334 (P-334) (RT = 3.12, λ_{max} = 334 nm) in *Anabaena* sp. HKAR-7 when treated with combined stress of PAR + UV-B + NH_4Cl for 21 days. Spectroscopic analysis of methanolic extracts of *Anabaena* sp. HKAR-7 showed absorption at 665 nm due to Chl *a*, at 470 nm due to carotenoids and absorption maxima for MAA at 334 ± 2 nm (Fig. 2a). Figure 2b depicts the absorption spectrum of partially purified MAA showing peak at 334 nm. HPLC chromatogram of purified MAA has been shown in Fig. 2c having the typical peak of MAA at RT of 3.12 min and Fig. 2d shows the absorption maximum for HPLC purified MAA P-334 at 334 nm. As mentioned earlier, HPLC purified MAA was utilized for production of protonated molecules by ESI–MS. Prominent ion peak of protonated molecules $[M+H]^+$ at *m/z* 346.8 was observed in ESI–MS analysis (Fig. 3). Identification of the purified MAA and its quantification was done as per the method described earlier (Sinha et al. 1999). Interestingly, cyanobacterial samples exposed under different experimental

Table 4 Effect of PAR, UV-B radiation and NH_4Cl (200 µM) on phycocyanin content in *Anabaena* sp. HKAR-7

Treatments	Phycocyanin (mg mL^{-1}) (mean ± SD)		
	Time (days)		
	6	15	21
Control	$0.36 \pm (0.014)^{NS}$		
PAR	$0.35 \pm (0.024)^{a}$	$0.35 \pm (0.029)^{ab}$	$0.26 \pm (0.024)^{b}$
PAR + NH_4Cl	$0.36 \pm (0.030)^{a}$	$0.31 \pm (0.035)^{a}$	$0.27 \pm (0.020)^{a}$
PAR + UV-B	$0.27 \pm (0.034)^{a}$	$0.22 \pm (0.030)^{a}$	$0.11 \pm (0.025)^{b}$
PAR + UV-B + NH_4Cl	$0.31 \pm (0.031)^{a}$	$0.26 \pm (0.029)^{a}$	$0.16 \pm (0.012)^{b}$

Results are expressed as means of three replicates. Similar letters represent homogeneous mean group ($P > 0.05$)

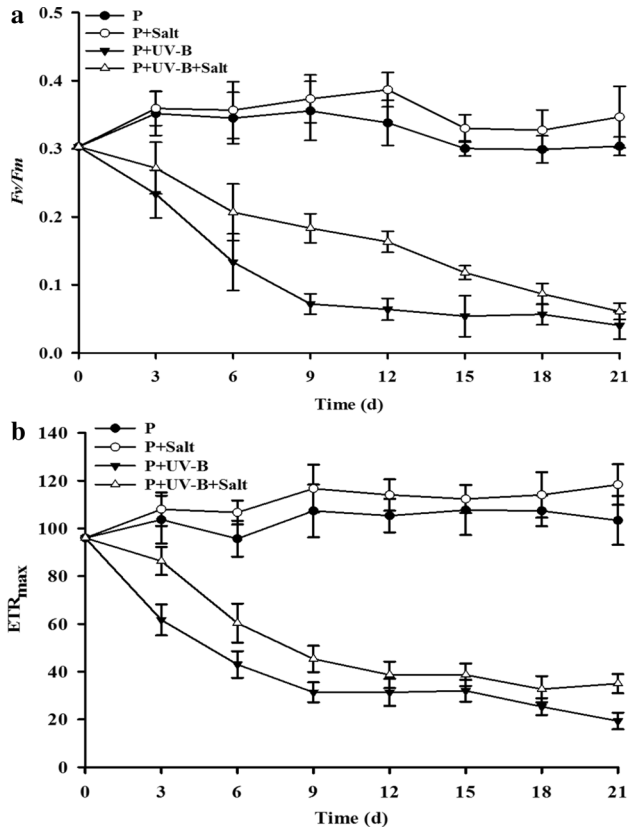

Fig. 1 Effect of PAR, UV-B radiation and NH_4Cl on maximum quantum yield (*Fv/Fm*) (**a**) and maximum electron transport rate (ETR_{max}) (**b**) in *Anabaena* sp. HKAR-7. *C* control, *P* PAR, Salt: NH_4Cl (200 µM). Results are expressed as means of three replicates. The error bars denote standard deviations of means (means ± S.D., n = 3)

conditions showed enhanced induction of MAA in the following increasing order i.e. PAR < PAR + NH_4Cl < PAR + UV-B < PAR + UV-B + NH_4Cl treatments. In *Anabaena* sp. HKAR-7 maximum induction of MAA P-334 was observed in the cyanobacterial samples exposed to combined stress of PAR + UV-B + NH_4Cl (1.378 µmol/g dry wt) for 21 days (Fig. 4).

Discussion

The physiological and biochemical response of any organism including cyanobacteria is greatly influenced by variation in their environment. For carrying out the process of photosynthesis and nitrogen fixation, cyanobacteria get exposed to high doses of damaging UVR (Balskus and Walsh 2010) which might result in photo-transformations in the genetic material (DNA) because of production of cyclobutane pyrimidine dimers, thymine-thymine pyrimidine-pyrimidone6–4) photoproducts and DNA–protein cross-links (Batista et al. 2009; Rajneesh et al. 2018; Pathak et al. 2019b). Activation of different lines of defense strategies including screening of UVR through UV-absorbing compounds such as MAAs increases resistance of cyanobacteria to high irradiances (Korbee-Peinado et al. 2004, 2005; Huovinen et al. 2006; Richa 2015; Rastogi et al. 2016). Photoprotective compounds, MAAs, not only play an important role in UVR screening but also act as antioxidant molecules, compatible solutes, intracellular nitrogen reservoir and aid in defense against thermal, desiccation and other stress conditions (Bandaranayake 1998; Oren and Gunde-Cimerman 2007; Rastogi et al. 2016; Richa et al. 2018). It has been found that low nitrogen nutrition results in decrement in the contents of Chl *a* and soluble proteins including RuBisCO in different cyanobacteria and algae (Beardall et al. 1991; Wulff et al. 2000). Supplementation of exogenous antioxidants and nitrogen source helps the organisms to overcome several abiotic stresses.

In the present study, certain doses of NH_4Cl (50, 200 and 500 µM), positively influenced the growth of cyanobacterium *Anabaena* sp. HKAR-7 as indicated by changes in Chl *a* content. Here, 200 µM concentration of NH_4Cl was found to be optimum as higher concentration of ammonium causes uncoupling of photophosphorylation in photoautotrophs and results in cellular toxicity, which becomes more pronounced under high light conditions (Britto and Kronzucker 2002; Zhu et al. 2000; Drath et al. 2008). Also, prolonged exposure to such combined stress (PAR + UV-B + NH_4Cl) generates ROS ($O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, $^1O_2$) which results in significant reduction in the growth of cyanobacterium with increasing duration of UV-B exposure and concentration of NH_4Cl. UV-B radiation exhibits detrimental effects on photosynthetic pigments which might be correlated to photoreduction of protochlorophyllide to chlorophyllide (Marwood and Greenberg 1996). Chlorophylls form complexes with proteins and lipids and thereby exist in a highly organized state. Hence, the decrement in Chl *a* content due to UVR exposure may be the result of degradation of lipids, proteins and their complexes associated with the thylakoid membrane (Prasad and Zeeshan 2005).

Carotenoids serve as important pigments which help in photoprotection against damaging effects of UVR. Increasing concentration of carotenoids in response to PAR + UV-B + NH_4Cl stress is in accordance with its role as ROS scavenger in photoautotrophs, hence, providing crucial defense mechanism against photooxidation (Vincent and Quesada 1994; Pattanaik et al. 2008). Enhanced biosynthesis of carotenoids aids in increased utilization of light in the low and middle regions of the PAR spectrum and help in quenching the active oxygen species and free radicals (Paerl et al. 1983; Götz et al.1999). In this study, carotenoids content increased initially to prevent cyanobacterial cells from photooxidation. Under prolonged UV-B exposure, cells generated more ROS that might be a reason for decreased

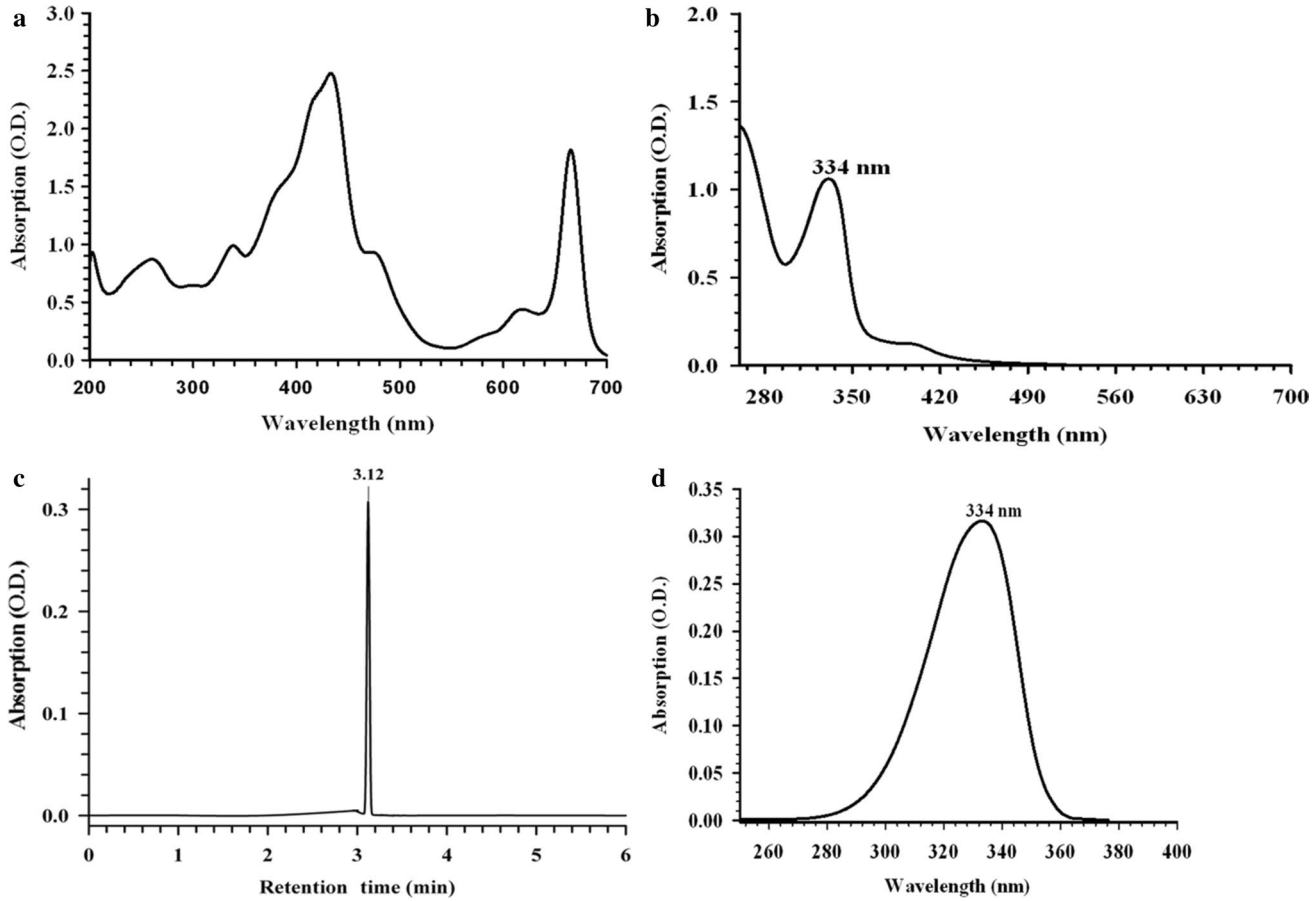

Fig. 2 Absorption spectrum of methanolic extract (**a**) and partially purified MAA (**b**). HPLC chromatogram of purified MAA showing the typical peak at RT of 3.12 min (P-334) (**c**) and absorption maximum for P-334 at 334 nm (**d**) in *Anabaena* sp. HKAR-7

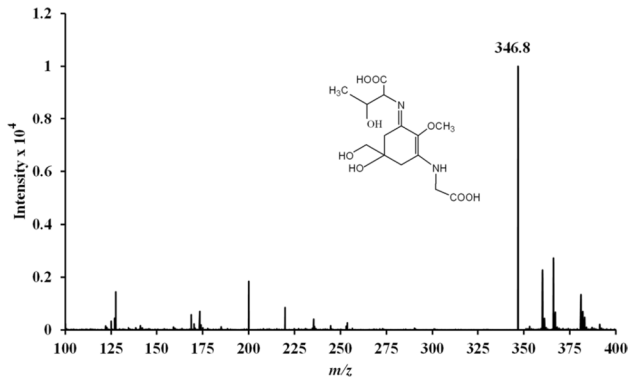

Fig. 3 Electrospray ionization-mass spectrometry (ESI–MS) of HPLC purified fraction of MAA, P-334 (*m/z* 346.8)

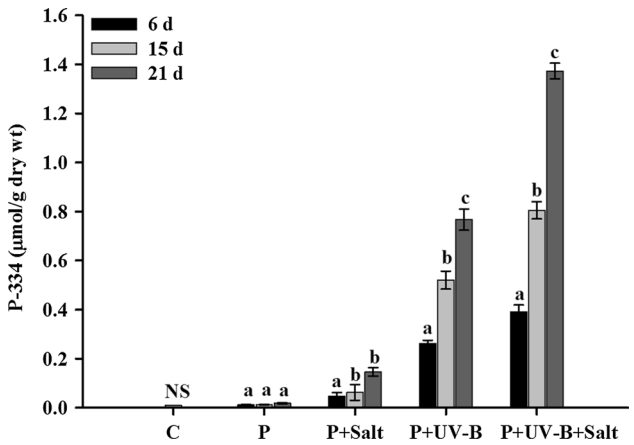

Fig. 4 Effect of PAR, UV-B radiation and NH_4Cl on induction of P-334 concentration in *Anabaena* sp. HKAR-7. Results are expressed as means of three replicates. Vertical bars indicate standard deviation of the means. Similar letters over bar represent homogeneous mean group ($P > 0.05$). *C* control, *P* PAR; Salt: NH_4Cl (200 µM)

biosynthesis of carotenoids via photosynthesis, leading to marked decrease in its content. Besides, this decrease in carotenoids synthesis was least in nitrogen supplemented samples, which explains that its synthesis was more favourable under surplus nitrogen availability. Decrement in

carotenoids content in turn affects PC, Chl *a* and thylakoid membrane adversely, resulting in the reduced photosynthetic efficiency of cyanobacteria. Phycobiliprotein in phycobilisomes are nitrogen storage compounds which funnel light energy to the underlying PSII reaction centres (Kannaujiya and Sinha 2015). Cyanobacterial phycobiliproteins are sensitive to degradation upon UV-B exposure as these are localized on the thylakoid's outer surface membrane (Donkor and Häder 1991; Kannaujiya and Sinha 2015), however, this damage was quite less in cyanobacteria which were exposed to UV-B radiation along with supplementation of NH_4Cl.

Damage to the photosynthetic apparatus on exposure to UV-B has been observed in several algae (Wulff et al. 2007; Bhandari and Sharma 2011). However, samples which were supplied with NH_4Cl along with UV-B treatment seem to generate a quick repair mechanism after removal of UV-B stress. In cyanobacteria, D1 and D2 proteins of reaction centres (PSII) are sensitive to UVR and were found to be replaced immediately after UVR exposure and such rapid turnover of proteins of PSII reaction centre helps the organisms in acclimatizing to the stressed environment (Sicora et al. 2006). Repair of the damaged PSII occurs via energetically costly process of protein synthesis (Lesser et al. 1996) which might be one of the reasons for the increased repair capacity of the cells supplemented with external nitrogen source and this explains the least depressed *Fv/Fm* and ETR_{max} values in the cyanobacterial samples exposed to UV-B + NH_4Cl. Phytoplankton show more sensitivity to UV-B radiation under nutrient-deficiency in comparison to nutrient-replete conditions and UV-B exposure under nutrient-deficiency damages the enzymes responsible for regulating the process of nitrate and ammonium uptake, hence, adversely affects the nitrogen metabolism (Döhler 1992; Lesser et al. 1994; Lohman et al. 1998). In this study also responses of cyanobacteria, mainly the recovery processes were modified during the UVR treatment on exogenous supplementation of NH_4Cl in a dose dependent manner.

Induction of light-dependent MAA biosynthesis was higher in UV-B radiation as compared to PAR in cyanobacteria (Sinha et al. 2002; Richa 2015). Similarly, PAR and PAR + NH_4Cl exposed cyanobacterial samples showed higher rate of *Fv/Fm* and ETR_{max} as compared to UVR exposed samples (PAR + UV-B and PAR + UV-B + NH_4Cl). MAA performs its photoprotective function by absorbing highly energetic UVR and dissipating it to the surroundings as heat (Conde et al. 2004). Here, UV-B radiation induced P-334 in a dose dependent manner with increased duration of exposure. Addition of NH_4Cl further enhanced MAAs biosynthesis and synergistically effected its induction along with UVR in *Anabaena* sp. HKAR-7 which was in accordance with the previous findings (Singh et al. 2008). Some studies have questioned the photoprotective role of MAA as its induction and accumulation was not observed in response to UVR or PAR exposure, also, it failed to provide complete protection to the organisms against UVR (Garcia-Pichel et al. 1993; Neale et al. 1998; Gröniger et al. 1999; Yakovleva and Titlyanov 2001). The accumulation and biosynthesis of MAA is not always attributed by solar radiation alone as several other abiotic stresses/factors such as salinity, temperature and availability of nutrients also induce its biosynthesis (Bandaranayake 1998; Dunlap and Shick 1998; Karsten and Wiencke 1999; Singh et al. 2020). Combined stress of PAR + UV-B + NH_4Cl significantly induced biosynthesis of P-334 in comparison to exposure of PAR, PAR + NH_4Cl, and PAR + UV-B indicating an MAA-specific induction which was triggered by exposure of PAR + UV-B + NH_4Cl. Induction of MAAs biosynthesis is dependent on the quality (wavelength) as well as duration of incident radiation (Karsten et al. 1998a, b; Karsten and Wiencke 1999; Franklin et al. 2001). This explains the reduced damage to the cyanobacterial photosynthetic apparatus on exposure to UV-B radiation which helped the cyanobacteria in maintaining the photosynthetic yield in spite of decrement in the Chl *a* content. However, role of repair mechanisms such as de novo synthesis of D1 and D2 protein of PSII and photoreactivation cannot be ruled out in the absence of UVR exposure. Photoreactivation helps in repair of damaged DNA by the enzyme "DNA photolyase" which utilize blue wavelength of solar radiation for correcting the modified nitrogenous bases of DNA to their normal forms (Senger 1982; Britt 1996; Todo et al. 1996; Sinha and Häder 2002; Zhang et al. 2013).

Basic skeleton of MAAs are made up of cyclohexenone and cyclohexenimine cores, which mainly consist of nitrogen and carbon. The requirement of nitrogen was completed by NH_4Cl. However, deprivation of carbon availability might limit the efficacy of the MAAs biosynthesis as it was found that photoheterotrophic growth condition was required in *Anabaena* sp. for synthesis of MAAs (Singh et al. 2014). There is possibility that cyanobacterium utilizes other cellular carbon compounds for MAAs biosynthesis. Nitrogen fixation is an energetically expensive physiological process, hence, cyanobacteria do not fix atmospheric nitrogen in the presence of available nitrogen (Pandey et al. 2018). In presence of exogenous nitrogen source cyanobacteria can allocate this energy in the biosynthesis of MAAs explaining higher MAA biosynthesis in presence of NH_4Cl in the present study. Results from present investigation may help in controlling the growth of harmful cyanobacteria in water bodies by reducing the nutrient availability and increasing the levels of UV-B exposure. Sustainable cultivation of cyanobacteria at commercial scale is the major limiting factor in their optimum utilization for the production of biofertilizers, energy and numerous secondary metabolites of nutritional and medicinal values. Optimization of exogenous supplementation of key elements such as nitrogen may

help in enhanced biomass production of cyanobacteria and can serve as sustainable agricultural practice for obtaining very high value cyanobacterial biomass.

Conclusion

Limited doses of external nitrogen in form of NH_4Cl supported the growth of *Anabaena* sp. HKAR-7 and aided the organism in tolerating the adverse effects of UV-B as indicated by its photosynthetic activity (*Fv/Fm* and ETR_{max}) and pigment composition. Our results suggest that in addition to quantity and quality of the incident radiation, nutrient availability (optimum dose of NH_4Cl) significantly enhanced the levels of MAAs in *Anabaena* sp. HKAR-7. Higher levels of photoprotective compound P-334 might play important role in protecting the cyanobacterium from lethal effects of prolonged UV-B exposure. Ammonium protected the cyanobacterium against lethal UVR not only by enhancing resistance by inducing MAA biosynthesis, but also by increased recovery of the crucial process of photosynthesis. These results can be used as one of the various ways for enhanced and sustainable production of value added compounds such as MAAs for their possible applications in cosmetics and pharmaceutical industries.

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interests.

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