


UV-B radiation and temperature stress-induced alterations in metabolic events and defense mechanisms in a bloom-forming cyanobacterium *Microcystis aeruginosa*

Piyooosh K. Babel¹ · Garvita Singh¹ · Anjali Singh² · Ashok Kumar¹ · Madhu B. Tyagi² · Rajeshwar P. Sinha³ 

Received: 12 August 2016/Revised: 20 September 2017/Accepted: 3 October 2017/Published online: 13 October 2017
© Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Kraków 2017

Abstract The present investigation is aimed to understand how a bloom-forming cyanobacterium *Microcystis aeruginosa* adapts to changing climatic conditions. The cyanobacterium was exposed to stresses of UV-B (2 Wm^{-2}) radiation and temperature ($45 \text{ }^\circ\text{C}$) for desired time intervals. Results showed that both the stresses affect growth and photosynthetic efficiency of *M. aeruginosa*. More than 50% loss of survival and content of photosynthetic pigments was noted after 4 h treatment of both the above stresses. Such changes were mainly due to the generation of reactive oxygen species which cause damage to proteins, DNA, lipids, and modulation of the membrane stability. An increase in the proline accumulation was noted in the cells which probably negates the harmful effects. In addition, activity of antioxidative enzymes namely, catalase, superoxide dismutase, ascorbate peroxidase, and peroxidase was induced by 1.5–3.0-fold on 3 h of UV-B and temperature treatment indicating their possible role in protection. Interestingly, induction of photoprotective compound, mycosporine-like amino acids (MAAs)

were also found under UV-B stress which might be an additional strategy of defense mechanism for the survival of the cyanobacterium. Analysis of photoprotective compound revealed shinorine as the main MAA synthesized by the cyanobacterium.

Keywords Abiotic stress · Antioxidants · Cyanobacterium · Mycosporine-like amino acids (MAAs) · *M. aeruginosa* · Reactive oxygen species (ROS)

Introduction

Cyanobacteria came in existence about 3.5×10^9 years ago (in Precambrian Era) when there was no oxygen and ozone shield. Under those anoxic conditions, they faced environmental extremes such as high temperature and intense solar radiation containing lethal UV radiation. Early life of cyanobacteria on Earth was influenced by these unfavorable factors (Schopf 2000; Herman 2010). At the end of the last century, many anthropogenic activities, such as the release of chlorofluorocarbons (CCl_3F and CCl_2F_2) and reactive nitrogen species (ONOO^- , NO and N_2O) into the atmosphere, have resulted in depletion of the ozone layer that absorbs the damaging ultraviolet radiation from the sun. As a consequence, risk of exposure of aquatic organisms to ultraviolet-B (UV-B; 280–315 nm) radiation and biologically effective temperature has increased (Herman 2010; Bais et al. 2014). In aquatic ecosystems, UV-B radiation, constitute less than 1% of the total solar energy, is considered as one of the most damaging environmental factors for photosynthetic organisms (Manney et al. 2011; Llabres et al. 2013).

Global warming, verified by increases in daily, seasonal, and annual mean temperature (Yamori et al. 2014), is

Communicated by Z. Miszalski.

Electronic supplementary material The online version of this article (doi:10.1007/s11738-017-2540-4) contains supplementary material, which is available to authorized users.

✉ Rajeshwar P. Sinha
r.p.sinha@gmx.net; rpsinhabhu@gmail.com

¹ School of Biotechnology, Banaras Hindu University, Varanasi 221005, India

² Department of Botany, MMV, Banaras Hindu University, Varanasi 221005, India

³ Laboratory of Photobiology and Molecular Microbiology, Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi 221005, India

expected to have intense and diverse impacts on all the biological systems including cyanobacteria. In recent decades, changes in climatic events have caused severe impacts and damage to ecosystem including primary producers such as cyanobacteria. *Microcystis aeruginosa* is one of the major bloom-forming cyanobacterial species, which forms noxious blooms in eutrophic freshwater reservoirs, ponds, and lakes, and causes negative impacts on the ecosystem. Few strains of *M. aeruginosa* are responsible for the production of potent toxins called microcystins. These formidable toxins cause human and livestock and poisoning. Optimal temperature required for photosynthesis and growth of harmful bloom causing cyanobacteria such as *Microcystis* have been found at or above 25 °C (Johnk et al. 2008). However, not much informations are available regarding the growth response of non-toxic and toxic strains of cyanobacteria with respect to increasing water temperature in an ecosystem. It is, therefore, important to assess the manner in which these cyanobacteria may adapt under climate change. These findings would also lead to questions related to the capacity of cyanobacteria to become dominant under future climate regimes.

An elevation in UV-B radiation and temperature can result in cell damage by directly affecting the biomolecules such as DNA, lipids, and proteins (Hargreaves et al. 2007; Häder et al. 2015). Heat stress affects the structure of thylakoid membrane, thereby hampering PS II activity, and resulting in inactivation of chloroplast enzymes followed by loss of chlorophyll (Ruelland and Zachowski 2010). Several studies have suggested the role of oxidative stress via reactive oxygen species (ROS) formation to be the possible cause of cell damage (Camejo et al. 2006; Naudts et al. 2014). UV-B-induced oxidative stress and heat may also significantly increase the membrane peroxidation leading to changes in the membrane thermostability (Mittler et al. 2012). It has also been reported that ROS produced in cyanobacteria due to high temperature and UV-B can cause double or single strand breaks in the DNA molecule (He and Häder 2002). The lifestyle of cyanobacteria enables them to adapt to different environmental conditions. Earlier studies have shown response of *Anabaena* species under UV-B radiation stress and it was found that a complex set of adaptive strategies have developed by these species to counteract UV-B radiation-induced damages (Babele et al. 2012; Singh et al. 2013). Another study on cyanobacterium *Nodularia spumigena* showed that concentrations of chlorophyll *a*, carotenoids, and total mycosporine-like amino acid were significantly higher in the UV-B treated cultures (Wulff et al. 2007). As *M. aeruginosa* is the most common and ubiquitous bloom-forming cyanobacterium around the world and forms massive blooms especially during summer months, a

period when they are exposed to both high temperature and intense solar UV radiation, it is expected that they may be well adapted to the above two abiotic stresses (Carmichael et al. 2001; Yakes et al. 2015). However, till date, there is paucity of information pertaining to the combined effects of elevated temperature along with UV-B radiation stress on *M. aeruginosa*. Furthermore, little, if any, information is available dealing with protection strategies adopted by this organism which enable it to form massive blooms under condition of elevated UV-B and temperature stress. Prompted by the gap of knowledge on the above aspects as well as world-wide abundance of *M. aeruginosa*, our interest aroused to select this species in the present study.

The goal of the present investigation was to explore (1) whether UV-B radiation and temperature exert any detrimental effect on growth and photosynthetic pigments of *M. aeruginosa*? (2) Do these stresses induce oxidative stress in this cyanobacterium, and (3) what kind of protective mechanisms operate under elevated UV-B during its growth and temperature stresses. Accordingly, the present study aims to analyze the effects of temperature and UV-B radiation on photosynthetic pigments, ROS content, lipid peroxidation, proline content, DNA damage, activity of antioxidative enzymes, and synthesis of photoprotective compounds (MAAs). This study may be useful in understanding the response of this toxic cyanobacterium towards abiotic stresses and strategies employed for survival and growth.

Materials and methods

Test organism and growth conditions

The cyanobacterium *M. aeruginosa* used in the present study was isolated during the month of May 2015 from a eutrophicated pond (Durgakund) situated in the central part of holy city Varanasi (25°21'N, 83°1'E, and ~ 76 m above mean sea level), India. The preliminary identification of the isolate was made by microscopic observations (Fig. S1A, B). Under microscope, well-defined thick-walled spherical cells were observed with characteristic gas vesicles and colorless mucilage. In addition, molecular approach mainly *16S rRNA* gene amplification with *Microcystis* sp. specific primers was employed for identification. Primer sequences for *16S rRNA* gene were; forward AGAGTTTGATCCTGGCTCAG, and reverse TACGGCTACCTTGTTACGA. The PCR was performed on a PTC-0200 DNA EngineTM Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., USA) with a thermal cycle of; 5 min at 95 °C followed by 35 cycles of 20 s at 95 °C, 30 s at 50 °C, and 1.5 min at 72 °C with a final step of 5 min at 72 °C. The identity of isolate was confirmed by sequencing of amplified *16S rRNA* amplicon and matching

with the GenBank database. Identification of the most similar 16S rDNA was done using the algorithm BLASTN programme. Sequence showed 99% similarity with the available nucleotide sequences of *M. aeruginosa* species (NCBI accession number JF799854).

BG 11⁺ (with combined inorganic nitrogen source) medium was used for routine growth of cultures under axenic conditions (Rippka et al. 1979) at 25 ± 2 °C in a culture room and illuminated with Sylvania 40 W T12 fluorescent lamp at an intensity of 14.4 ± 1 Wm⁻² for a 14/10 h light/dark cycle. Exponentially growing cultures were used for performing all the experiments.

Source and mode of UV-B and temperature treatment

UV-B radiation treatment was given by a UV-B lamp (Cat No. 3-4408, Fotodyne Inc., USA) giving its main output at 312.67 nm. Distance between the UV-B lamp and the sample was adjusted in a manner to get the desired intensity of UV-B (2 Wm⁻²) to the culture suspension. A specially fabricated chamber equipped with an exhaust fan (to avoid overheating) was used for irradiation. Black-Ray J-221, long-wave ultraviolet intensity meter (UVP, Inc., San Gabriel, CA, USA), was used for measuring the UV-B intensity. At global level UV-B in solar radiation averaged about 2.3 Wm⁻²; hence, 2 Wm⁻² of UV-B radiation intensity was selected in the experiments for the exposure of cultures (Madronich et al. 1998). Furthermore, the low intensity of UV-B shows delayed effects on cyanobacteria. We had been performing experiments using 2 – 2.5 Wm⁻² of dose in all the experiments (Kumar et al. 2004, 2016). It is pertinent to mention that the intensity of UV-B used (2 Wm⁻²) in the present work is much higher than the ambient intensity of UV-B in the solar radiation; however, the present study deals with short-term experiments; nevertheless, the results may be extrapolated to long-term studies as the organisms are exposed to lower doses of solar UV-B radiation throughout whole day for months. For UV-B exposure, 500 mL exponentially grown culture of *M. aeruginosa* was taken and from which 100 mL each was transferred in five 120 mm sterile Petri dishes (diameter 120 mm, with open lids). Four plates were exposed to UV-B radiation (2 Wm⁻²) together with fluorescent light for desired time intervals (1, 2, 3, and 4 h) and one plate was exposed to fluorescent light alone which served as control. Cultures were gently stirred magnetically to facilitate uniform exposure. Similarly, for temperature treatment, four culture flasks (250 mL capacity), each containing 100 mL culture suspension, were placed inside a water bath at a temperature of 45 °C. One flask containing 100 mL culture was kept in culture room at 25 ± 2 °C and served as control. Selection of temperature

(45 °C) was based on the data of field study conducted during the months of May and June (2015) for the collection of *M. aeruginosa* from eutrophicated pond (Durgakund) wherein a temperature of 42–45 °C was recorded during the time of study.

After exposure to UV-B radiation/temperature, required volume of samples was withdrawn at desired time intervals and was used for the estimation of survival, growth, photosynthetic pigments, ROS generation, lipid peroxidation, proline content, DNA strands break, activity of antioxidant enzymes, and MAAs content. The log phase cultures having an initial dry weight of ~ 0.15 mg mL⁻¹ were taken for performing the experiments unless otherwise stated. Each experiment was repeated thrice under identical conditions.

Determination of percent survival, growth, and pigment content

Percent survival, chlorophyll *a*, and phycocyanin content were determined by the methods described earlier (Singh et al. 2013). Absorbance was recorded in a double beam UV-Vis spectrophotometer (Shimadzu 1800-UV, Shimadzu Corp., Japan). The raw spectra were analyzed using UV Probe version software (Shimadzu Corp.). All spectroscopic measurements were performed at room temperature.

Determination of ROS contents

To determine the generation of ROS in UV-B and temperature exposed and control cultures, 5 μM of DCFH-DA (2',7'-dichlorofluorescein-diacetate) (final concentration in ethanol) was added to the cultures and incubated for 1 h in the dark on a shaker at 37 °C. As temperature and UV irradiation rapidly auto-oxidizes or hydrolyzes DCFH-DA, it cannot be added before stress exposure. After 1 h of incubation, samples were subjected to spectrofluorometric analysis in a spectrofluorometer (Varian Instruments, CA, USA) and fluorescence microscopy (Nikon Eclipse 50i, Japan) at an excitation wavelength of 485 nm and an emission band between 500 and 600 nm.

Determination of lipid peroxidation and proline content

Measurement of MDA formed as an end product of lipid peroxidation was done following the method of Li et al. (2006) with some modifications. UV-B and temperature treated and control cultures (10 mL) were harvested by centrifugation ($8000 \times g$ for 10 min at 4 °C). The cell homogenate was weighed and mixed with 2.5 mL of 0.67% (w/v) TBA (2-thiobarbituric acid) made in 15%

trichloroacetic acid (TCA). Subsequently, the mixture was heated in boiling water for 30 min and then quickly cooled on ice bath. After centrifugation ($10,000\times g$ for 10 min), the absorbance of the supernatant was recorded at 450, 532, and 600 nm. The MDA concentration was estimated using the equations: $C_0 = 6.45 (OD_{532} - OD_{600}) - 0.56OD_{450}$ and $C = C_0/FW$; here, OD = measured absorbance at λ , respectively; FW = fresh weight of the cell homogenate; and C = MDA concentration ($\mu\text{mol g}^{-1}$ FW).

Proline content of the cells was calculated according to Bates et al. (1973) with little modifications. Treated and control cultures (10 mL) were centrifuged at 4 °C ($8000\times g$ for 10 min). The cells extract was prepared with 5 mL of 3% sulfosalicylic acid in boiling water for 10 min followed by centrifugation. Supernatant (2 mL) containing proline was pipetted and treated with ice cooled acetic acid (2 mL) and acid-ninhydrin (2 mL) followed by incubation at 100 °C for 30 min. The reaction was terminated in an ice bath, and then, the mixture was extracted with 4 mL of toluene. Absorbance was recorded at 520 nm. The standard curve for proline was prepared by dissolving proline in 3% sulfosalicylic acid to cover the concentration range of 0.5–10 mg mL⁻¹. Absorbance for all the experiments was recorded at room temperature in a double beam spectrophotometer (Shimadzu 1800-UV, Shimadzu Corp., Japan). The raw spectra were analyzed using UV Probe version software (Shimadzu Corp.).

Test of DNA strands breaks

For determination of DNA strands break, fluorometric analysis of DNA unwinding (FADU) was performed as per the method demonstrated by Rastogi et al. (2011). To check the extent of DNA breaks in UV-B irradiated cells (designated as P-sample), two additional sets of untreated control samples (namely B and T) with identical OD at 665 nm were taken as reference. Cells from all the three samples were harvested by centrifugation at $10,000\times g$ for 10 min. The cell pellet was washed with TE buffer and resuspended in solution A (50 mM Tris, pH 8.0; 50 mM Na₂EDTA; 1 M NaCl). Subsequently, sarkosyl solution (10% *N*-lauroylsarcosine, 10 mM Tris-HCl, pH 8.0; 20 mM EDTA) was added to the samples and kept at 4 °C for 2 h. After centrifugation ($10,000\times g$, 10 min), the pellet was washed twice with TE buffer. Then, the pellet was resuspended in solution B (50 mM Tris, pH 8.0; 50 mM Na₂EDTA; 25% sucrose) to attain a final volume of 184 μL . 20 μL of 50 mg mL⁻¹ lysozyme was added to the suspension, and the mixture was incubated for 60 min at 37 °C to destroy the cell wall. Subsequently, 30 μL 10% SDS, 9 μL of 10 mg mL⁻¹ proteinase K, 10 μL 4 M NaCl, and 47 μL TE buffer were added to make a final volume of 300 μL . Samples were incubated at 37 °C for 60 min for

achieving the complete lysis of the cells. Finally, 300 μL of NaOH (0.1 M) was added to all three samples and subjected to different unwinding protocols as described by Rastogi et al. (2011). After completion of unwinding protocols, 20 μL of DNA probe, i.e., Hoechst 33258 (bis-benzimide) (20 mM) in 0.6 M phosphate buffer (pH 7.6) was added to all the samples and centrifuged ($10,000\times g$, 5 min). One millilitre supernatant of each sample was taken to measure the fluorescence intensity in Varian Cary Eclipse spectrofluorometer (Varian Instruments, CA, USA) with an excitation wavelength of 485 nm and an emission band between 500 and 600 nm. All fluorescence measurements were performed at room temperature. The percentage fraction (% *F*) of dsDNA was calculated according to He and Häder (2002). The experimental protocol for temperature stress was similar to those used for UV-B.

Estimation of antioxidative enzyme activity

Activities of antioxidative enzymes were determined using standard methods as described previously (Singh et al. 2013). Absorbance was recorded in a double beam spectrophotometer. The raw spectra were analyzed using UV Probe version software (Shimadzu Corp.). All spectroscopic measurements were performed at room temperature.

Extraction and characterization of mycosporine-like amino acids (MAAs)

Extraction and characterization of MAAs were done as described previously (Singh et al. 2013; Richa and Sinha 2015). Briefly, cells were extracted in 100% methanol and kept at 4 °C overnight. After brief sonication of cell extract, it was centrifuged at $8000\times g$ for 10 min and the absorbance of clear supernatant was recorded between 200 and 400 nm to confirm the presence of MAAs. Methanolic extract was air dried and dissolved in MQ water and filtered solution was subjected to HPLC analysis for further characterization and partial purification. Furthermore, partially purified MAAs was characterized with ESI-MS and FTIR analyses as described earlier (Singh et al. 2013).

Statistical analysis

For statistical reliability, all the experiments were repeated thrice under identical conditions. A one-way ANOVA (analysis of variance) was applied to confirm the significance of data according to Duncan's multiple range test (DMRT) at $P \leq 0.05$. SPSS-16 software was used for DMRT. Unless otherwise stated, values are the mean \pm SD ($n = 3$).

Results

Effect of temperature and UV-B radiation on survival

Effects of temperature and UV-B radiation on *M. aeruginosa* were assessed by examining the percentage survival and chlorophyll *a* content after the time intervals of 1, 2, 3, and 4 h. Values obtained were compared with the control (untreated) cultures. Cells exposed to UV-B and temperature stress for 4 h in the presence of fluorescent light showed a drastic decline in the survival (50% loss), the decline being more pronounced with UV-B (Fig. S-2A). A linear decrease in Chl *a* (Fig. S-2B) and phycocyanin (Fig. S-2C) content was also observed with increasing duration of temperature and UV-B stress. Percent decrease in Chl *a* content after 1 h of temperature and UV-B exposure was lower (7 and 10%, respectively) but increased to 51 and 47% after 4 h of continuous exposure (Fig. S-2B). A drastic reduction in phycocyanin content was also observed after 4 h of exposure to temperature and UV-B; the content decreased to approximately 60 and 40%, respectively (Fig. S-2C).

Effects on ROS generation

UV-B radiation exposure of cultures along with enhanced temperature resulted in increased ROS level more specifically after 1 h. Microscopic analysis of DCF fluorescence in the cells revealed that the generation of ROS is triggered after 1 h of exposure and becomes more prominent with increasing duration of exposure (Fig. 1). These results match with the results of spectrofluorometric analysis (Fig. S3). When the ROS generation was compared between the two oxidative stresses, temperature exposure showed 1.58-fold increase in ROS generation at 2–4 h of exposure, whereas UV-B exposure resulted in 1.25-fold increase at 2 and 3 h and reached to 1.55-fold after 4 h as compared to control. It is evident from the microscopic and fluorescence spectroscopic observations that both the oxidative stresses trigger production of ROS, induction being almost similar under both the stresses.

Effects on lipid peroxidation and proline content

Peroxidation of lipid was induced by temperature and UV-B stress. MDA content in temperature and UV-B treated cultures increased with increasing duration of exposure as compared to the control cultures. Lipid peroxidation increased by more than 2.5- and 1.9-fold in UV-B and temperature treated cultures, respectively, after 4 h of continuous exposure (Fig. 2a). As such, UV-B radiation

caused more damage to lipids in comparison to temperature.

Proline concentration also showed approximately two-fold higher level following 3 h of temperature and UV-B treatment as compared to control. However, some decline in proline concentration was observed at 4 h (10% decline) under both temperature and UV-B stress (Fig. 2b). This suggests that exposure to UV-B and temperature stress beyond 3 h does not favor proline accumulation.

Effects on DNA strand breaks

Analysis of DNA strand breaks showed significant effect on DNA damage under both the stresses. Breakage in the DNA strand showed a linear increase with increasing duration of temperature and UV-B exposure. DNA damage increased by more than twofold after 4 h of continuous exposure of cultures to temperature and UV-B stress (Fig. 3). However, DNA damage was not affected significantly after 1 h of temperature and UV-B exposure as compared to control cultures.

Effects on antioxidative enzymes

It is evident from the results that activity of antioxidative enzymes is induced markedly under the stress of temperature and UV-B radiation most probably to overcome the harmful effects. About 2.9- and 3.2-fold increase in the activity of SOD were recorded after 3 h of continuous exposure of UV-B and temperature treated cultures, respectively, as compared to control cultures. After 4 h, the activity began to decline but maintained higher level than the control culture (Fig. 4a). Catalase activity of control cultures was 0.052 units mg^{-1} protein which increased to 0.12 and 0.10 units in UV-B and temperature exposed cultures at 3 h. After 4 h exposure of cultures to UV-B and temperature, the activity decreased to 0.092 and 0.087 units mg^{-1} protein, respectively (Fig. 4b). Peroxidase activity was 0.008 units in control cultures but increased to 0.02 and 0.018 units after 3 h of UV-B and temperature treatment, respectively; the activity declined to 0.018 and 0.015 units after 4 h (Fig. 4c). The pattern of APX enzyme activity was more or less similar to those of peroxidase activity. Control cultures showed an activity of 0.0013 units which increased to 0.0021 and 0.0019 units after 3 h of exposure of cultures to UV-B and temperature, respectively. Activity in 4 h exposed cultures to UV-B and temperature came down to 0.0016 and 0.0014 units, respectively, which were almost similar to the control cultures (Fig. 4d).

Effects on mycosporine-like amino acids (MAAs)

Spectroscopic analysis of methanolic extract of cultures showed a prominent peak of UV-absorbing compound

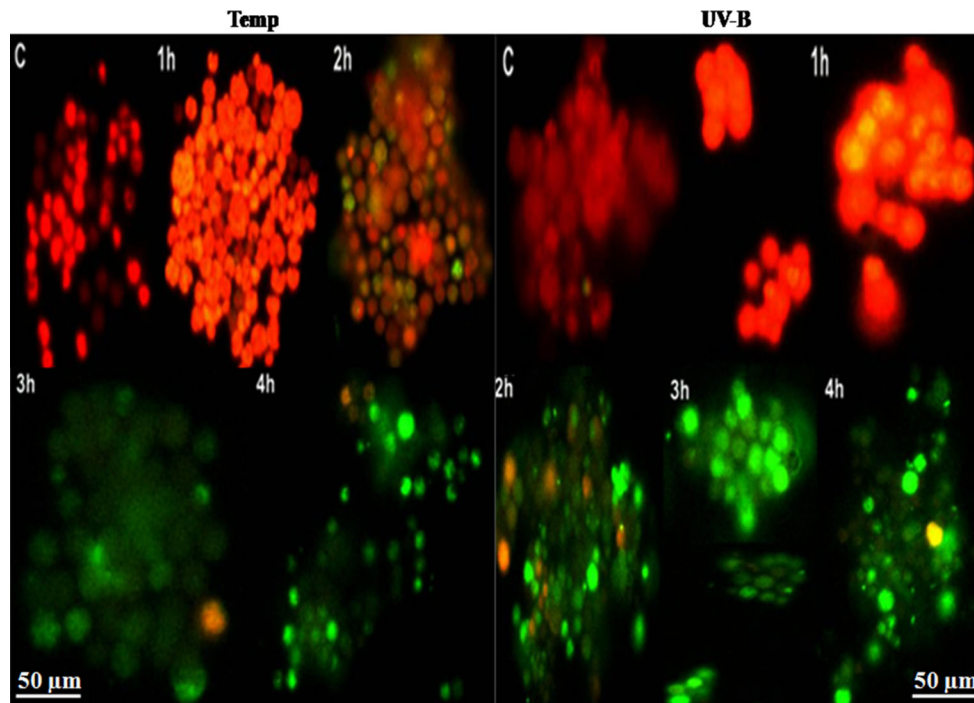
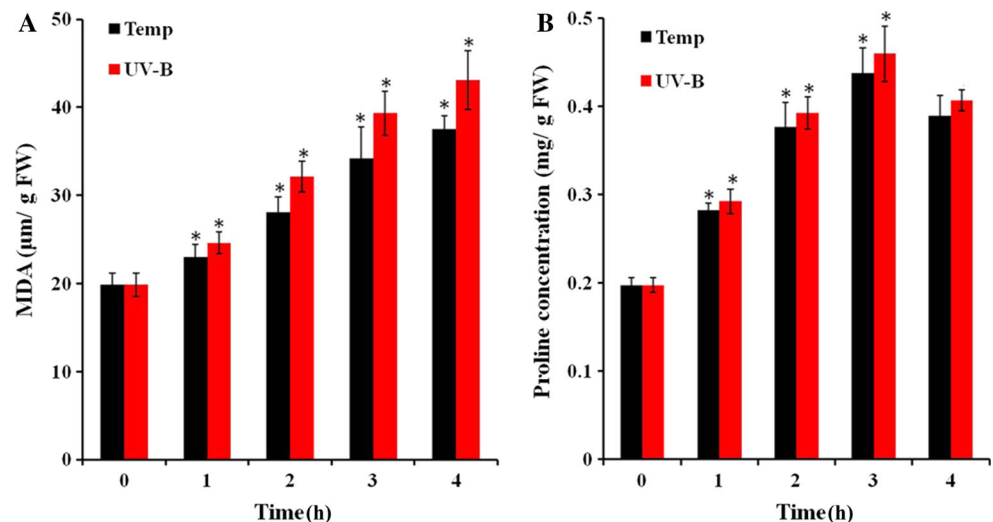


Fig. 1 Fluorescence microphotographs of *M. aeruginosa* cells showing intracellular red (no ROS generation) and green (ROS production) DCF fluorescence after temperature (45 °C), and UV-B (2 W m^{-2})

exposure for varying time intervals. C—without any exposure; 1, 2, 3, and 4 h exposure time. Scale bars indicate 50 μm

Fig. 2 Estimation of **a** lipid peroxidation and **b** proline content in *M. aeruginosa* cells exposed to temperature and UV-B radiation for varying time periods. Values are mean \pm SD ($n = 3$) and asterisks indicate significant differences from 0 h ($*P < 0.05$). One-way ANOVA was used according to Duncan's multiple range test (DMRT) at $P \leq 0.05$



(absorbance between 320 and 350 nm) suggesting the presence of colorless MAAs in the test organism. Test of induction under both the stresses showed insignificant effect of temperature even after 4 h of exposure; however, UV-B exposure to cultures showed a marked impact on the induction of MAAs. There was approximately twofold induction in the amount of MAAs after 3 h of UV-B

exposure (Fig. 5). Notably, further increase in the duration of UV-B exposure (4 h) led to decrease in MAAs content, although the content was still higher than the basal level of MAAs. Further analysis by HPLC showed the presence of a few peaks at 334 nm (retention time 2.1–2.9 min) which resembled to standard MAAs (Fig. 6a). Major peak (fraction) of MAAs (retention time 2.9 min) was collected and

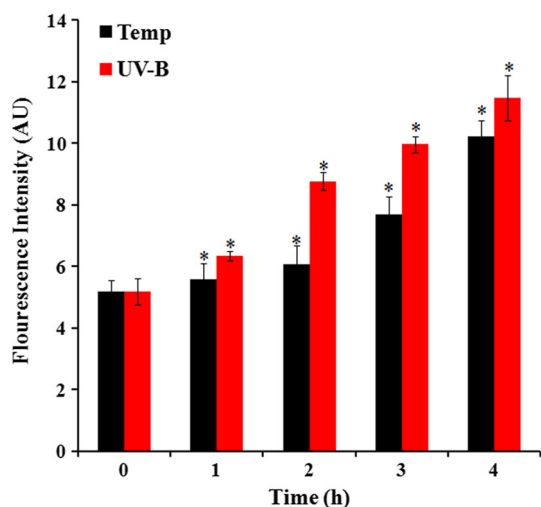


Fig. 3 Effects of temperature and UV-B radiation on DNA strand breaks in *M. aeruginosa* cells with varying time duration. Values are mean \pm SD ($n = 3$) and asterisks indicate significant differences from 0 h ($*P < 0.05$). One-way ANOVA was used according to Duncan's multiple range test (DMRT) at $P \leq 0.05$

subjected to HPLC analysis again together with standard which showed a single prominent peak corresponding to the MAA shinorine (Fig. 6b).

Partially purified MAA showed a peak of m/z 321.1¹⁺ on ESI-MS analysis which further confirmed the MAA as shinorine (of shinorine with retention time 2.4 min, MW-334) was noted (Fig. 6c). Furthermore, six prominent bands similar to those reported for shinorine were observed in FTIR results of the fractions containing KBr pellets. Bands of 1385.2 cm^{-1} may be assigned for the presence of carboxylic groups and that of 1634.8 cm^{-1} for NH_2 . Band of 2927.1 cm^{-1} could be assigned for side chain vibrations consisting of C-H stretching and indicating the presence of NH_2^+ . Wave band of 3448 cm^{-1} may be assigned to OH^- group (Fig. 6d).

Discussion

In the present study, impacts of UV-B radiation and temperature on the bloom-forming cyanobacterium *M. aeruginosa* were analyzed. Results show that abiotic stresses namely UV-B radiation and high temperature affect growth and a number of metabolic processes of the test organism. UV-B radiation and elevated temperature induce oxidative stress due to the production of ROS. Such a conclusion is

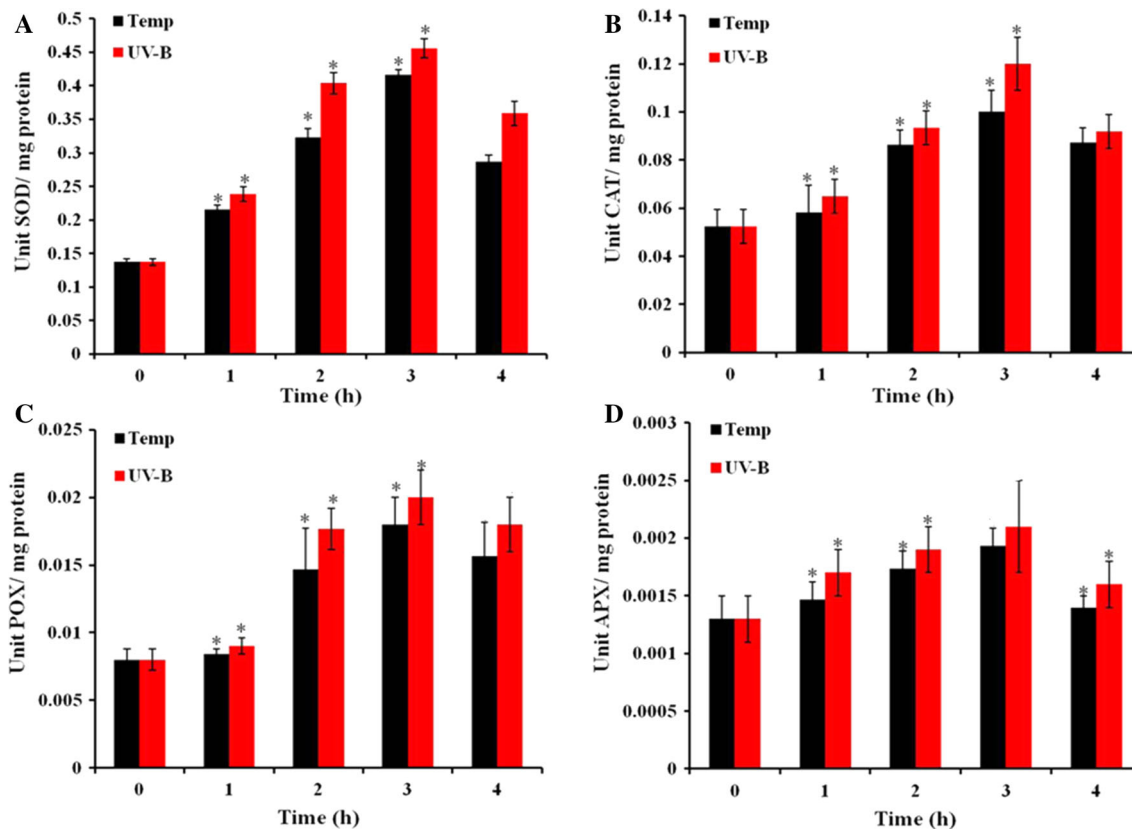


Fig. 4 Activity of antioxidative enzymes in *M. aeruginosa* exposed to temperature and UV-B radiation for varying time periods. **a** SOD, **b** CAT, **c** POD, and **d** APX. Values are mean \pm SD ($n = 3$) and

asterisks indicate significant differences from 0 h ($*P < 0.05$). One-way ANOVA was used according to Duncan's multiple range test (DMRT) at $P \leq 0.05$

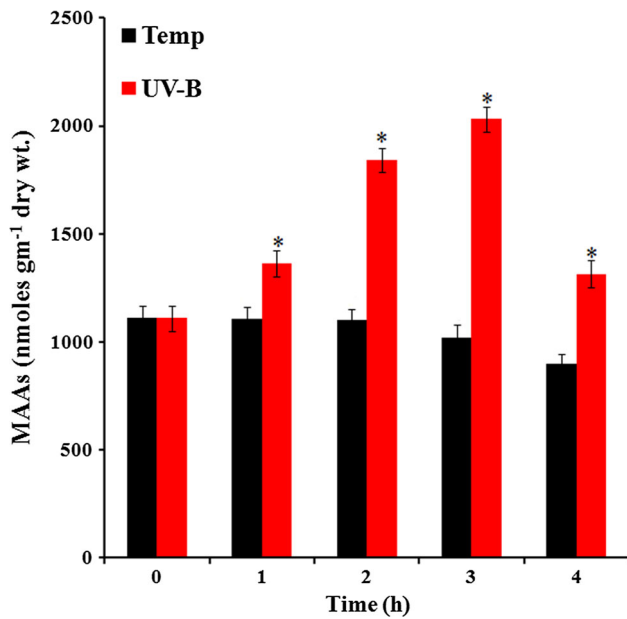


Fig. 5 Effects of temperature and UV-B radiation on MAAs concentration in *M. aeruginosa* cells exposed to varying time periods. Values are mean \pm SD ($n = 3$) and asterisks indicate significant differences from 0 h ($*P < 0.05$). One-way ANOVA was used according to Duncan's multiple range test (DMRT) at $P \leq 0.05$

based on the fact that formation of ROS increased with increasing duration of stress. Usually, multifaceted regulatory pathways are expressed in cyanobacteria which involve ROS (Jones 2006; Foyer and Noctor 2009). Excessive production of ROS is known for decreased photosynthetic activity due to the destruction of D1 protein that inhibits photosynthetic efficiency (Wendehenne et al. 2004; Wang et al. 2011). Induced oxidative stress is believed to be the primary incident, and when organism undergoes environmental stresses such as temperature and UV stress, it acts as an alert signal. ROS generated due to oxidative stress are known to react with important structural and functional biomolecules such as DNA, proteins, and lipids, resulting in their damage and cell death (Van Breusegem and Dat 2006). Findings of this study also showed a similar sequence of events due to increased ROS generation as is evident from DNA damage and lipid peroxidation. These events probably affect the metabolic processes as decrease in survival and photosynthetic pigments, namely, chlorophyll *a* and phycocyanin, was evident after stress. Protective response is triggered by ROS which allows cell to adapt to the oxidative stress or induce cell cycle arrest or even death (Shirkey et al. 2000; Apel

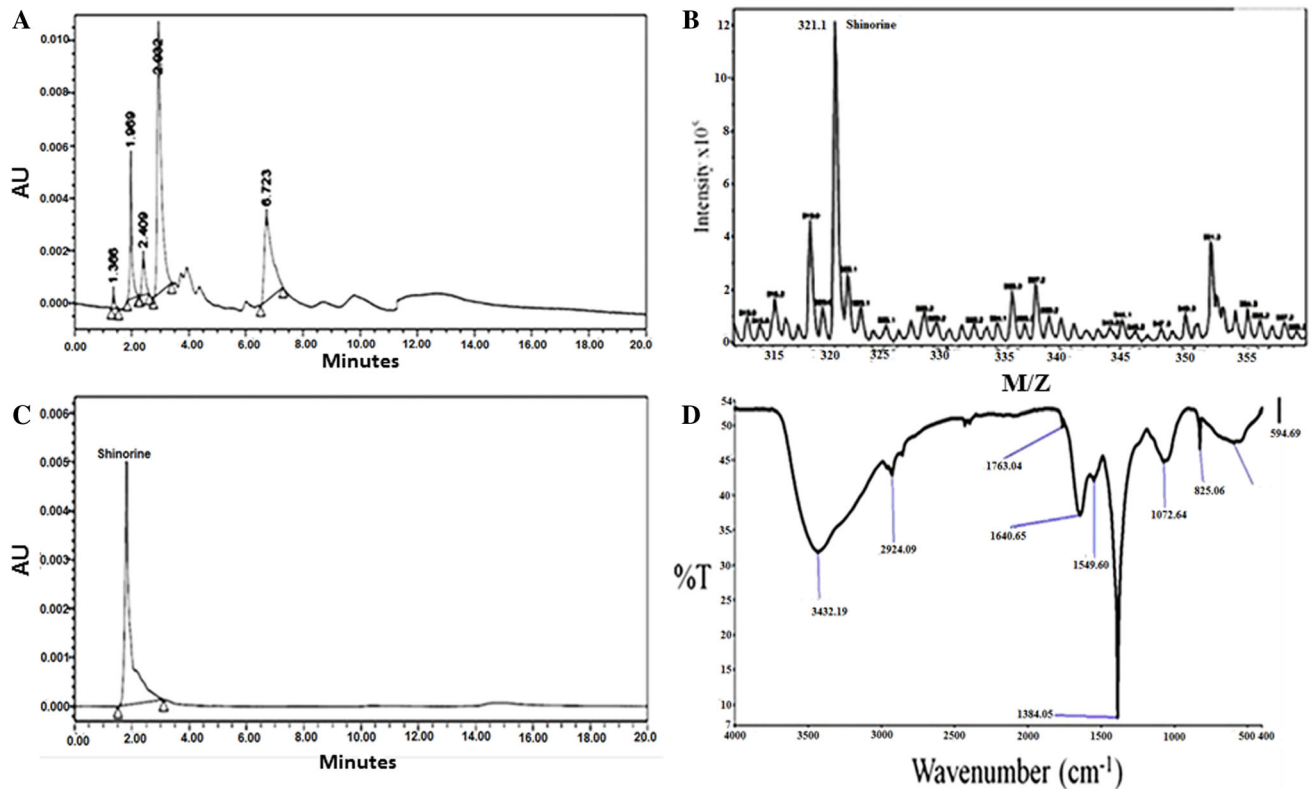


Fig. 6 Characterization of the MAA shinorine employing HPLC, ESI-MS, and FTIR from the cell extracts of *M. aeruginosa* **a** HPLC chromatogram showing the presence of different MAAs, **b** HPLC purified shinorine, peak appearing at 2.0–2.5 min resembles to the

MAA shinorine (SH), **c** electro spray ionization–mass spectrometry (ESI-MS), and **d** Fourier transform infrared radiation (FTIR) spectroscopy showing functional groups present in purified fraction from *M. aeruginosa* showing peaks resembling to MAA shinorine

and Hirt 2004). A decrease of about 50% in chlorophyll *a* and 40% in phycocyanin content under both UV-B and temperature stress was observed as compared to untreated (control) cells of *M. aeruginosa*. However, pronounced effect was noted on phycocyanin as it acts as photosensitizers. It appears that chlorophyll and phycocyanin are the prime targets of UV-B and temperature and their loss results in gross changes of the metabolic machinery.

It is believed that thylakoid membranes have relatively more content of polyunsaturated fatty acids (PUFA) for the proper photosynthetic activities (Allakhverdiev et al. 2009). Unfortunately, PUFAs are vulnerable to oxidative damages in the form of lipid peroxidation. Lipid peroxidation has been reported to proceed via a series of reactions and generate lipid peroxyl radicals unless tocopherol or ascorbate come into existence and take part to scavenge these harmful radicals (Halliwell and Gutteridge 1999). Changes in total MDA production have also been used as one of the indicators of lipid peroxidation in the present study (Kyungsil et al. 2004). Results of this study showed a significant increase in MDA content (up to twofold) following exposure of cultures to both temperature and UV-B stress. Henceforth, decrement in chlorophyll *a* content may be attributed to the membrane destabilization by increased lipid peroxidation (Li et al. 2006). Results found in this study are in agreement with the previous reports (Marwood and Greenberg 1996) wherein photoreduction of protochlorophyllide under stress conditions has been implicated.

Proline production is believed to be one of the first metabolic responses against any environmental stress (Hare and Cress 1997). Enhanced accumulation of proline maintains osmotic balance, and provides defense to enzymes as well as biological membranes (Basak et al. 2001). It is believed that proline is capable of detoxifying free radicals by forming a stable complex (Backor et al. 2004). In this study, the intracellular level of proline increased with increasing duration of stress, suggesting that elevation of oxidative stress resulted in simultaneous increase of intracellular proline. However, there is a check point and after prolonged duration of stress exposure proline accumulation declines and fails to provide protection against the stress-induced effects. This is also evident from the findings of the present study wherein increase in proline level till 3 h of stress exposure showed protective role, but decrease in its level beyond 3 h resulted in the decrease of protective role. Most probably, certain other mode of defense mechanisms becomes operative when the level of proline drops in the cell. Besides the loss of survival, decrease in pigment content and induction of lipid peroxidation, UV-B, and temperature stress also caused DNA strand break. Fluorometric analysis of DNA unwinding (FADU) elicited significant decrease in dsDNA with

increasing duration of UV-B and temperature exposure. Damage to DNA is considered as one of the key factors for cell death including necrosis and apoptosis (Pourzand and Tyrrell 1999; Gao et al. 2008).

Findings of the present study suggest the existence of two lines of defense mechanisms under temperature and UV-B stress, one being enzymatic and another non-enzymatic mediated through synthesis of a special class of secondary metabolites namely MAAs. It was noted that exposure of cultures to UV-B (2 Wm^{-2}) radiation and temperature ($45 \text{ }^\circ\text{C}$) up to 3 h resulted in a significant increase in the level of antioxidative enzymes activity. Beyond 3 h of exposure, there was decrease in activity which might be due to the loss of survival of the organism. UV-B radiation-induced increase in the biosynthesis of secondary metabolites such as MAAs and antioxidative enzymes such as SOD, CAT, GPX, and POD has been reported earlier in *Anabaena* sp. (Singh et al. 2013). Further studies are required to characterize these transitions where lipid peroxidation and proline accumulation act as signal for the induction of either enzymatic or non-enzymatic pathways of defense mechanisms.

Microcystis species are frequently challenged by UV and temperature stress in their natural habitats. The presence of UV-absorbing compounds (MAAs) may thus play a vital role in protection against environmental stresses. MAAs have been isolated from different organisms including those growing in marine, freshwater, or terrestrial habitats. Among MAAs, shinorine, porphyra-334, and mycosporine-glycine are most common and reported from different species of cyanobacteria (Carreto and Carignan 2011). Results of this study show that *M. aeruginosa* possesses relatively higher concentration of MAAs identified as shinorine even in the absence of any stress, but the concentration increased by more than twofold under UV-B stress. Our finding is similar to the earlier report where approx. 4–5-fold increase was reported in *Anabaena* species under UV-B stress (Singh et al. 2013). Differences in the level of MAAs induction may be due to the differences in genetic machinery of different cyanobacterial genera and/or the presence of protective mechanism other than MAAs under various stresses (Ding et al. 2013). This explanation is strongly supported from the fact that the photoreceptor-encoding genes are present adjacent to the MAA cluster in *Anabaena*, whereas they are absent in *Microcystis* species (Hu et al. 2015), and that MAAs may not be solely involved in protection against UV-B stress is also evident from the report that *Microcystis* species in general has mosaic-like distribution of MAAs genes to produce MAAs (Hu et al. 2015). Some other aspects such as self-shading through colony formation, production of microcystin, and buoyancy regulation may represent additional means for UV adapting *Microcystis* (Ding et al.

2013). Altogether, it may be concluded that MAAs partly play role in protection especially against UV-B radiation considering its twofold induction. However, there is need of further work to assign the exclusive role of MAAs as sunscreen pigment *Microcystis* species.

Conclusions

Till date, *Microcystis* species have been extensively explored for its toxic bloom-forming capacity and their harmful effects on human and other organisms. This study is probably the first to deal with the effects of abiotic stresses namely UV-B radiation and temperature to which blooms of *Microcystis* species are frequently exposed. Results show that increase in temperature and intensity of UV-B radiation severely affects growth and causes damage to various physiological processes. The primary target of both the stresses seems to be key biomolecules like lipids, proteins, and DNA; their damages probably cause disruption of metabolic events. Both temperature and UV-B cause oxidative stress resulting in enhanced generation of ROS. Increased ROS content causes damage to the membrane stability due to peroxidation of lipids and also damages DNA as a result of break in the double helical structure. However, due to enhanced accumulation of proline and activity of antioxidative enzymes, *M. aeruginosa* mitigates harmful effects of oxidative stress. Accumulation of proline acts as osmotic protectant and antioxidative enzymes scavenge the toxic ROS radicals. This enzymatic mode of protection strategy may partly allow growth and survival of the organism under abiotic stresses. In addition, induction of photoprotective compound shinorine (MAAs) during UV-B radiation stress may also play an important role in combating the damaging effects. To our knowledge, isolation and characterization of shinorine from *M. aeruginosa* are the first report and its role in UV-B stress protection has been implicated. In conclusion, findings of this study clearly show that both enzymatic and non-enzymatic mode of protection mechanism operate and may be beneficial for the bloom-forming *M. aeruginosa* in overcoming the natural load of stress to which they are frequently challenged. However, further studies are still needed to gain a better understanding of the cumulative effects of temperature and UV-B radiation as well as other modes of protective mechanism(s) operative in *M. aeruginosa* under the scenario of global warming.

Author contribution statement PKB: designed, performed the experiments, and wrote the MS. GS: performed the experiments and helped in drafting the paper. AS: helped in performing experiments and drafting the paper. AK: generated the idea, designed the study, and edited the

MS. MBT: evaluated the data and edited the MS. RPS: evaluated the data, helped in drafting the paper, and edited the MS.

Acknowledgements This work was supported by the project grant sanctioned to AK by the Department of Science and Technology, Govt. of India, New Delhi (SR/SO/PS-49/09). Thanks are due to IIT-BHU for providing facilities for the characterization of MAAs. PKB is grateful to the UGC, New Delhi, for the award of JRF.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

- Allakhverdiev SI, Los DA, Murata N (2009) Regulatory roles of photosynthesis of unsaturated fatty acids in membrane lipids. In: Wada H, Murata N (eds) Lipids in photosynthesis: essential and regulatory functions. Springer, Dordrecht, pp 373–388
- Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative and signal transduction. *Ann Rev Plant Biol* 55:373–399
- Babele PK, Singh G, Tyagi MB, Sinha RP, Kumar A (2012) Ultraviolet-B radiation effects on cyanobacteria and the role of sunscreen pigments in its protection. *Phykos* 42:1–13
- Backor M, Fahselt D, Wu CT (2004) Free proline content is positively correlated with copper tolerance of the lichen photobiont *Trebouxia erici* (chlorophyta). *Plant Sci* 167:151–157
- Bais AF, McKenzie RL, Bernhard G, Aucamp PJ, Ilyas M, Madronich S, Tourpalia K (2014) Ozone depletion and climate change: impacts on UV radiation. *Photochem Photobiol Sci* 14:19–52
- Basak M, Sharma M, Chakraborty U (2001) Biochemical responses of *Camellia sinensis* (L) O. Kuntze to heavy metal stress. *J Environ Biol* 22:37–41
- Bates LS, Waldren RP, Tear ID (1973) Rapid determination of free proline for water-stress studies. *Plant Soil* 39:205–207
- Camejo D, Jimenez A, Alarcon JJ, Torres W, Gomez JM, Sevilla F (2006) Changes in photosynthetic parameters and antioxidant activities following heat-shock treatment in tomato plants. *Funct Plant Biol* 33:177–187
- Carmichael WW, Azevedo SMFO, An JS, Molica RJR, Jochimsen EM, Lau S, Rinehart KL, Shaw GR, Eaglesham GK (2001) Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. *Environ Health Persp* 109:663–668
- Carreto JI, Carignan MO (2011) Mycosporine-like amino acids: relevant secondary metabolites. Chemical and ecological aspects. *Mar Drugs* 9:387–446
- Ding Y, Song L, Sedmak B (2013) UV-B radiation as a potential selective factor favoring microcystin producing bloom-forming cyanobacteria. *PLoS One* 8:e73919. doi:10.1371/journal.pone.0073919
- Foyer CH, Noctor G (2009) Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. *Antioxid Redox Signal* 11:861–905
- Gao K, Li P, Watanabe T, Helbling EW (2008) Combined effects of ultraviolet radiation and temperature on morphology, photosynthesis and DNA of *Arthrospira (Spirulina) platensis* (cyanophyta). *J Phycol* 44:777–786
- Häder DP, Williamson CE, Wangberg SA, Rautio M, Rose KC, Gao K, Helbling EW, Sinha RP, Worrest R (2015) Effects of UV

- radiation on aquatic ecosystems and interactions with other environmental factors. *Photochem Photobiol Sci* 14:108–126
- Halliwell B, Gutteridge JMC (1999) Free radicals in biology and medicine, 3rd edn. Oxford University Press Inc, New York, p 107
- Hare PD, Cress WA (1997) Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regul* 21:79–102
- Hargreaves A, Taiwo FA, Duggan O, Kirk SH, Ahmad SI (2007) Near-ultraviolet photolysis of b-phenylpyruvic acid generates free radicals and results in DNA damage. *J Photochem Photobiol B Biol* 89:110–116
- He YY, Häder DP (2002) Reactive oxygen species and UV-B: effect on cyanobacteria. *Photochem Photobiol Sci* 1:729–736
- Herman JR (2010) Global increase in UV irradiance during the past 30 years (1979–2008) estimated from satellite data. *J Geophys Res* 115:D04203. doi:10.1029/2009JD012219
- Hu C, Voller G, Submuth R, Dittmann E, Kehr JC (2015) Functional assessment of mycosporine-like amino acids in *Microcystis aeruginosa* strain PCC 7806. *Environ Microbiol* 17:1548–1559. doi:10.1111/1462-2920.12577
- Johnk KD, Huisman J, Sharples J, Sommeijer B, Visser PM, Strooms JM (2008) Summer heat waves promote blooms of harmful cyanobacteria. *Glob Change Biol* 14:495–512
- Jones DP (2006) Redefining oxidative stress. *Antioxid Redox Signal* 81:1865–1879
- Kumar A, Tyagi MB, Jha PN (2004) Evidences showing ultraviolet-B radiation-induced damage of DNA in cyanobacteria and its detection by PCR assay. *Biochem Biophys Res Commun* 318:1025–1030
- Kumar J, Babel PK, Singh D, Kumar A (2016) UV-B radiation stress causes alterations in whole cell protein profile and expression of certain genes in the rice phyllospheric bacterium *Enterobacter cloacae*. *Front Microbiol* 7:1–14. doi:10.3389/fmicb.2016.01440
- Kyungsil C, Pauli S, Marianne P (2004) Oxidative stress tolerance in the filamentous green algae *Cladophora glomerata* and *Enteromorpha ahlnneriana*. *J Exp Mar Biol Ecol* 298:111–123
- Li M, Hu CW, Zhu Q, Chen L, Kong Z, Liu Z (2006) Copper and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in the microalga *Pavlova viridis* (Prymnesiophyceae). *Chemosphere* 62:565–572
- Llabres M, Agusti S, Fernandez M, Canepa A, Maurin F, Vidal F, Duarte CM (2013) Impact of elevated UVB radiation on marine biota: a meta-analysis. *Glob Ecol Biogeog* 22:131–144
- Madronich S, McKenzie RL, Bjorn O, Caldwell MM (1998) Changes in biologically active ultraviolet radiation reaching the Earth's surface. *J Photochem Photobiol B Biol* 46:5–19
- Manney GL, Santee ML, Rex M, Livesey NJ, Pitts MC, Veefkind P, Nash ER, Wohltmann I, Lehmann R, Froidevaux L, Poole LR, Schoeberl MR, Haffner DP, Davies J, Dorokhov V, Gernandt H, Johnson B, Kivi R, Kyro E, Larsen N, Levelt PF, Makshtas A, McElroy CT, Nakajima H, Parrondo MC, Tarasick DW, von der Gathen P, Walker KA, Zinoviev NS (2011) Unprecedented arctic ozone loss in 2011. *Nature* 478:469–475
- Marwood CA, Greenberg BM (1996) Effect of supplementary UV-B radiation on chlorophyll synthesis and accumulation of photosystems during chloroplast development in *Spirodela oligorrhiza*. *Photochem Photobiol* 64:664–670. doi:10.1111/j.1751-1097.1996.tb03121.x
- Mittler R, Finka A, Goloubinoff P (2012) How do plants feel the heat? *Trends Biochem Sci* 37:118–125
- Naudts K, Van den Berge J, Farfan E, Rose P, AbdElgawad H, Ceulemans R, Janssens IA, Asard H, Nijs I (2014) Future climate alleviates stress impact on grassland productivity through altered antioxidant capacity. *Env Exp Bot* 99:150–158
- Pourzand C, Tyrrell RM (1999) Apoptosis, the role of oxidative stress and the example of solar UV radiation. *Photochem Photobiol* 70:380–390
- Rastogi RP, Singh SP, Hader DP, Sinha RP (2011) Ultraviolet-B-induced DNA damage and photorepair in the cyanobacterium *Anabaena variabilis* PCC 7937. *Env Exp Bot* 74:280–288
- Richa Sinha RP (2015) Biochemical characterization of sunscreens mycosporine-like amino acids from two *Nostoc* species inhabiting diverse habitats. *Protoplasma* 252:199–208
- Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Appl Microbiol* 111:1–61
- Ruelland E, Zachowski A (2010) How plants sense temperature. *Env Exp Bot* 69:225–232
- Schopf JW (2000) The fossil record: tracing the roots of the cyanobacterial lineage. In: Whitton BA, Potts M (eds) *The ecology of cyanobacteria*. Kluwer Academic Publishers, Dordrecht, pp 13–35
- Shirkey B, Kovarcik DP, Wright DJ, Wilmoth G, Prickett TF, Helm RF, Gregory EM, Potts M (2000) Active Fe-containing superoxide dismutase and abundant *sodF* mRNA in *Nostoc commune* (Cyanobacteria) after years of desiccation. *J Bacteriol* 182:189–197
- Singh G, Babel PK, Sinha RP, Tyagi MB, Kumar A (2013) Enzymatic and non-enzymatic defense mechanisms against ultraviolet-B radiation in two *Anabaena* species. *Process Biochem* 48:796–802
- Van Breusegem F, Dat JF (2006) Reactive oxygen species in plant cell death. *Plant Physiol* 141:384–390
- Wang G, Chen L, Hao Z, Li X, Liu Y (2011) Effects of salinity stress on the photosynthesis of *Wolffia arrhiza* as probed by the OJIP test. *Fresenius Environ Bull* 20:432–438
- Wendehenne D, Durner J, Klessig DF (2004) Nitric oxide: a new player in plant signaling and defense responses. *Curr Opin Plant Biol* 7:449–455
- Wulff A, Mohlin M, Sundback K (2007) Intra-specific variation in the response of the cyanobacterium *Nodularia spumigena* to moderate UV-B radiation. *Harmful Algae* 6:388–399
- Yakes BJ, Handy SM, Kanyuck KM, DeGrasse SL (2015) Improved screening of microcystin genes and toxins in blue-green algal dietary supplements with PCR and a surface plasmon resonance biosensor. *Harmful Algae* 47:9–16
- Yamori W, Hikosaka K, Way DA (2014) Temperature response of photosynthesis in C₃, C₄, and CAM plants: temperature acclimation and temperature adaptation. *Photosynth Res* 119:101–117