Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(1):362-371

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Isolation and partial purification of scytonemin and mycosporine-like amino acids from biological crusts

Jainendra Pathak, Richa, Rajneesh, Arun S. Sonker, Vinod K. Kannaujiya and Rajeshwar P. Sinha*

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

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ABSTRACT

During the last decade, scytonemin and mycosporine-like amino acids (MAAs) have gained considerable attention as an efficient natural photoprotectants against damaging ultraviolet (UV) radiation. In the present investigation biological crusts from different natural habitats such as roof top, bark of Mangifera indica, usar land, window ledge and agriculture field were examined for the presence of photoprotective compounds such as scytonemin and MAAs. These habitats represent diverse environments having high solar irradiance, high temperature, desiccation and nutrient depleted conditions. Biological crusts primarily composed of cyanobacteria such as Scytonema sp., Lyngbya sp. and Nostoc sp. Chlorophyll, carotenoids, scytonemin and MAAs were analyzed using absorption spectroscopic analysis. Identification and partial purification of scytonemin and MAAs were done by HPLC analysis which revealed the presence of scytonemin and MAAs in all the samples. Scytonemin had a retention time ranging from 1.43-3.85 min with absorbance maxima at 252, 278 and 386 nm and peaks of MAAs had a retention time ranging from 1.99-7.19 min with corresponding absorbance maxima at 309, 310, 330 and 332 nm corresponding to mycosporine-taurine, mycosporine-glycine, asterina-330 and palythinol respectively. Based on these findings, we conclude that photoprotective compounds such as scytonemin and MAAs may play a vital role not only in photoprotection but also in the survival and sustainability of cyanobacterial life, which are dominant organisms of most of biological crusts samples, in adverse environmental conditions such as under high solar irradiances, temperature, desiccation and nutrient depletion.

Keywords: Biological crusts, Cyanobacteria; High-performance liquid chromatography (HPLC); Scytonemin; Ultraviolet radiation (UVR).

INTRODUCTION

Depletion of ozone layer has resulted in an increase in the solar UV radiation on the Earth's surface which may cause several harmful effects thereby adversely affecting the existence and survival of all living organisms [1, 2]. Absorption of solar radiation to perform photosynthesis and nitrogen fixation exposes cyanobacteria to lethal ultraviolet-B (UV-B; 280–315 nm) radiation [3]. The highly energetic UV-B radiation affects biomolecules such as DNA and proteins directly as well as indirectly [4-7]. Direct effect includes the denaturation of DNA and RNA whereas indirect effects include production of reactive oxygen species [8, 9]. Several defense strategies have been evolved by cyanobacteria to counteract the damaging effects of UV radiation such as migration, mat formation, synthesis of antioxidants, presence of specific active repair mechanisms and UV-absorbing compounds [10, 11]. The UV-absorbing/screening compounds such as mycosporine-like amino acids (MAAs) and scytonemin have received considerable importance for their potential role in UV photoprotection [2, 12]. Scytonemin, a dimer of indolic and phenolic subunits, is a yellow-brown lipid soluble pigment located in the extracellular polysaccharide sheath of about 300 cyanobacterial species. Purified scytonemin has maximum UV absorption at 384 ± 2 nm, although it may also absorb significantly at 252, 278 and 300 nm [2, 13]. The main peak of scytonemin lies in the UV-C region, but

the additional peaks in the UV-B and UV-A regions are protective against UV radiation which is important in the current atmosphere which lacks UV-C. Scytonemin exists in oxidized (Mw 544 Da) as well as reduced (Mw 546 Da) form depending on the redox and acid-base conditions during the process of extraction [14]. Metabolites of aromatic amino acid biosynthesis are thought to be precursors of scytonemin biosynthesis, which can be induced by high photon fluence rate [14]. Scytonemin is highly stable against different stresses and performs its UV-absorbing/screening activity without any further metabolic investment [15]. The UV-sunscreen role of scytonemin has been well demonstrated in the terrestrial cyanobacteria [2, 8, 16]. Scytonemin prevent cellular damage by acting as an antioxidant against UV-induced production of ROS [8, 17].

MAAs are small (<400 Da), colorless, water-soluble compounds characterized by a cyclohexenone or cyclohexenimine chromophores conjugated with the nitrogen substituent of an amino acid or its imino alcohol [18, 19]. Several characteristics of MAAs, such as strong UV-absorption maxima between 310 and 362 nm, high molar extinction coefficients (ε = 28100-50 000M^{-1} cm^{-1}), photostability against several abiotic stressors and distilled and sea water in presence of photosensitizers give strong evidence for the photoprotective role of MAAs [20-23]. The role of MAAs as antioxidant in scavenging the UV-induced production of ROS has been worked out by several researchers [24-28]. There is still a great deal of controversy concerning the precise mechanisms of MAAs biosynthesis. The biosynthesis of MAAs has been suggested to occur *via* the first part of the shikimate pathway [10]. 3- dehydroquinate formed during the early stages of the shikimate pathway serves as the precursor for the synthesis of fungal mycosporines [29] and MAAs via gadusols [18, 30]. The primary MAA mycosporine-glycine thus synthesized by shikimate pathway is then transformed by chemical and/or biochemical conversions into other secondary MAAs [31]. The synthesis of MAAs occurs in bacteria, cyanobacteria, phytoplankton and macroalgae but not in animals, due to lack of shikimate pathway, the predicted pathway for their biosynthesis [30, 32]. MAAs have also been shown to be highly resistant against abiotic stressors such as temperature, UV radiation, various solvents and pH [20, 23]. Because of its potential UV-absorbing/screening capacity as well as numerous other biological applications [33, 34], MAAs and scytonemin may be biotechnologically exploited for pharmaceutical and cosmetic applications [35]. The main objective of this study was to screen and partially purify photoprotective compounds, scytonemin and MAAs from biological crusts collected from various natural habitats each representing a stressed environmental condition.

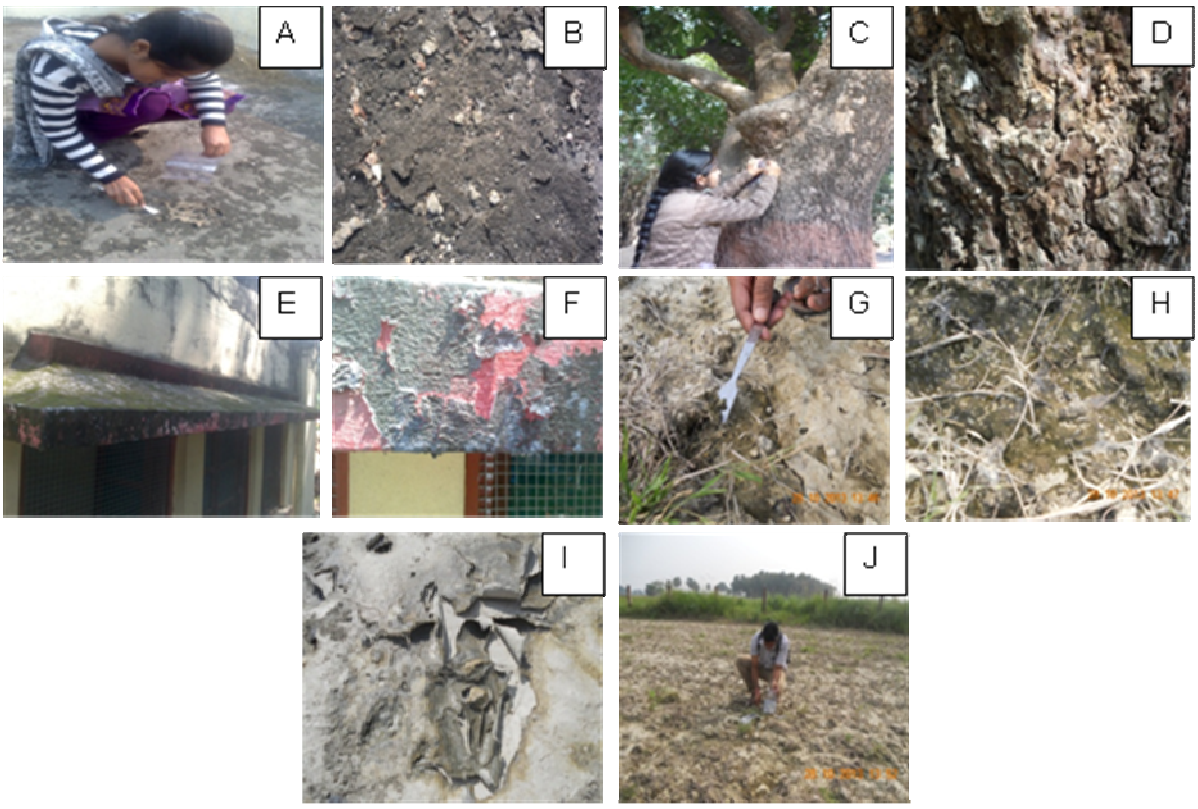

Fig. 1: Collection of cyanobacterial samples from different stressed habitats such as roof top (A and B), Bark of *Mangifera indica* (C and D), Window ledge (E and F), Department of Botany, Banaras Hindu University, Varanasi, and agriculture field (G and H), and Usar land (I and J), Maunath Bhanjan, India

EXPERIMENTAL SECTION

2.1 Collection site and sample collection

Samples were collected aseptically from five different sites namely roof top, Dept. of Botany, BHU, bark of *Mangifera indica*, BHU, window ledge, Dept. of Botany, BHU, agriculture field, Maunath Bhanjan and usar land, Maunath Bhanjan (Fig. 1). Varanasi is situated between 25° 28' North, 82° 96' East, in the Eastern part of Uttar Pradesh and Maunath Bhanjan is located between the 26° North, 87° East, about 120 km from Varanasi. Biological crusts were scraped from the surfaces with a sterile plastic scalpel. Samples were immediately placed into a sterile plastic bag and transported to the laboratory for further analyses. Digital images of sampling sites were recorded using a digital camera. Light intensity, UV-A, UV-B radiation, temperature and relative humidity at sites were recorded with a Lutron LX-101 lux meter, Solatech solarmeter, 5.0, 6.0digital thermometer and hygrometer respectively.

2.2 Culture and identification of organism

The collected crusts were soaked with sterile distilled water for 2 h and cleaned repeatedly with sterile distilled water. To examine the presence of different algae in the crust, a pinch of the rehydrated crust was observed in compound microscope. The organisms appearing in the cultures were examined using Image binocular microscope and photographed using Dewinter-2011 scientific digital camera and analysed with Dewinter software. The organisms belonging to cyanobacteria were identified following standard monographs and related articles [36, 37].

2.3 Pigment analysis

Pigment profile of crust was analyzed taking equal amount of crust (5 mg) from each sample, extracted in 90 % methanol (v/v) and absorption spectra of all samples were measured in a double beam spectrophotometer (U-2910, UV/VIS, Hitachi, Tokyo, Japan) in the wavelength range of 200-800 nm using quartz cuvettes. The raw data were transferred to a microcomputer and peaks were analyzed with software provided by the manufacturer. Quantity of chlorophyll *a* was estimated using the extinction coefficient of Mackinney [38] and Dere et al. [39] and the amount of total carotenoid was determined following Davis [40] and Dere et al. [39]. The cellular scytonemin content was calculated using the trichromatic equation following Gracia-Pichel and Castenholz [14] from 10 mg dry weight of cells. MAAs concentration in different crust samples were compared with the help of its relative absorbance at 330 nm.

2.4 Extraction of scytonemin

Scytonemin was extracted from crusts in 1:1 (v/v) methanol:ethyl acetate by overnight incubation at 4 ˚C followed by sonication (2011-Sonic, cycle 30%, Power 40%) for 4 min. After centrifugation (10,000g for 5 min), supernatants were evaporated in a vacuum evaporator at 38 ˚C and redissolved in 500 µL of 1:1 (v/v) methanol:ethyl acetate. Subsequently, the samples were filtered through 0.22 µm pore-sized sterilized microcentrifuge syringe-driven filter before being subjected to HPLC analyses.

2.5 Extraction of MAAs

MAAs was extracted from crusts in 2.0 ml of 100 % (v/v) methanol (HPLC-grade) by overnight incubation at 4 °C. After extraction, the aliquots were centrifuged (10,000g for 5 min) and supernatants (methanolic extracts) were evaporated to dryness at 45 °C and redissolved in 1 mL of double-distilled water and absorption spectrum was recorded. Thereafter, the samples were filtered through 0.2 µm pore-sized microcentrifuge filters and subsequently subjected to HPLC analysis.

2.5 HPLC analysis

Partially purified scytonemin and MAAs were analysed using a HPLC system (Waters 2998, Photodiode Array, pump L-7100, USA) equipped with a Licrospher RP 18 column and guard (5 µm packing; 250 mm x 4 mm inside diameter). For scytonemin samples (10 µL) were injected into the HPLC column through an auto-injector i.e Waters 717 plus autosampler. Elution was at a flow rate of 1.0 mL min^{-1} using the mobile phase of solvent A (ultra pure water) and solvent B (acetonitrile–methanol–tetrahydrofuran, 75:15:10, v/v). The 30 min gradient elution programme was set with 0–15 min linear increase from 15 % solvent A to 100 % solvent B, and 15–30 min at 100 % solvent B. The detection wavelength was at 376 nm. PDA scan wavelength was from 250 to 750 nm. Scytonemin was identified by its characteristic absorption maxima in the solvent corresponding to the appropriate retention time [2, 41]. Analyses of MAAs were done by injecting the samples (50 µl) into the HPLC column through a Waters 717 plus autosampler. The wavelength for detection was 330 nm, the mobile phase was 0.02 % acetic acid (v/v) in double-distilled water, at a flow rate of 1.0 ml min^{-1}[19]. The MAAs were identified by comparing the absorption spectra and retention times.

RESULTS

3.1 Environmental parameters, nature of the crust and algal identification

Varanasi lies in the dry humid ecoclimatic zone of the gangetic plain at an altitude of about 78 m encountering three characteristically distinct seasons namely, rainy (mid June-mid October), summer (March-June), Winter (Nov-Feb) with wide variations in temperature, solar radiations and relative humidity prevailing in all the seasons . Warm rainy season is marked by torrential rainfall with intermittent breaks, receiving around 90 % of annual rainfall possessing average day temperature, relative humidity and solar radiation in the range of 28-32 ˚C, 71-85 % and 259-335 cal cm^{-2} day^{-1} respectively. Virtually the rainy season is covering a period of the wettest months of July, August and September each receiving 18-42 % of annual rainfall, is the growing season for the cyanobacterium whereas the summer season receiving an average solar radiation around 367 cal cm^{-2} day^{-1} with maximum value upto 437 cal cm^{-2} day^{-1} and less than 5 % of the annual rainfall is a season with an increase in temperature up to 43 ˚C particularly in the month of May. The winter season receives low amount of rainfall and low temp. There is not much significant variation in the climatic conditions of Varanasi and Maunath Bhanjan. The environmental data in summer months shows light intensity varies from 41.56 to 45.34 w/m^2, UV-A 3.99 to 4.10 mw/cm^2, UV-B 0.43 to 0.47 mw/cm^2, temperature 40-45 ˚C and relative humidity 75- 85 % . The nature of crusts in all the five samples was greenish to brown in colour. In all the biological crusts filamentous cyanobacteria were in abundance. Species composition in the crusts varied with the amount of solar exposure and type of environmental stress present in the habitat.

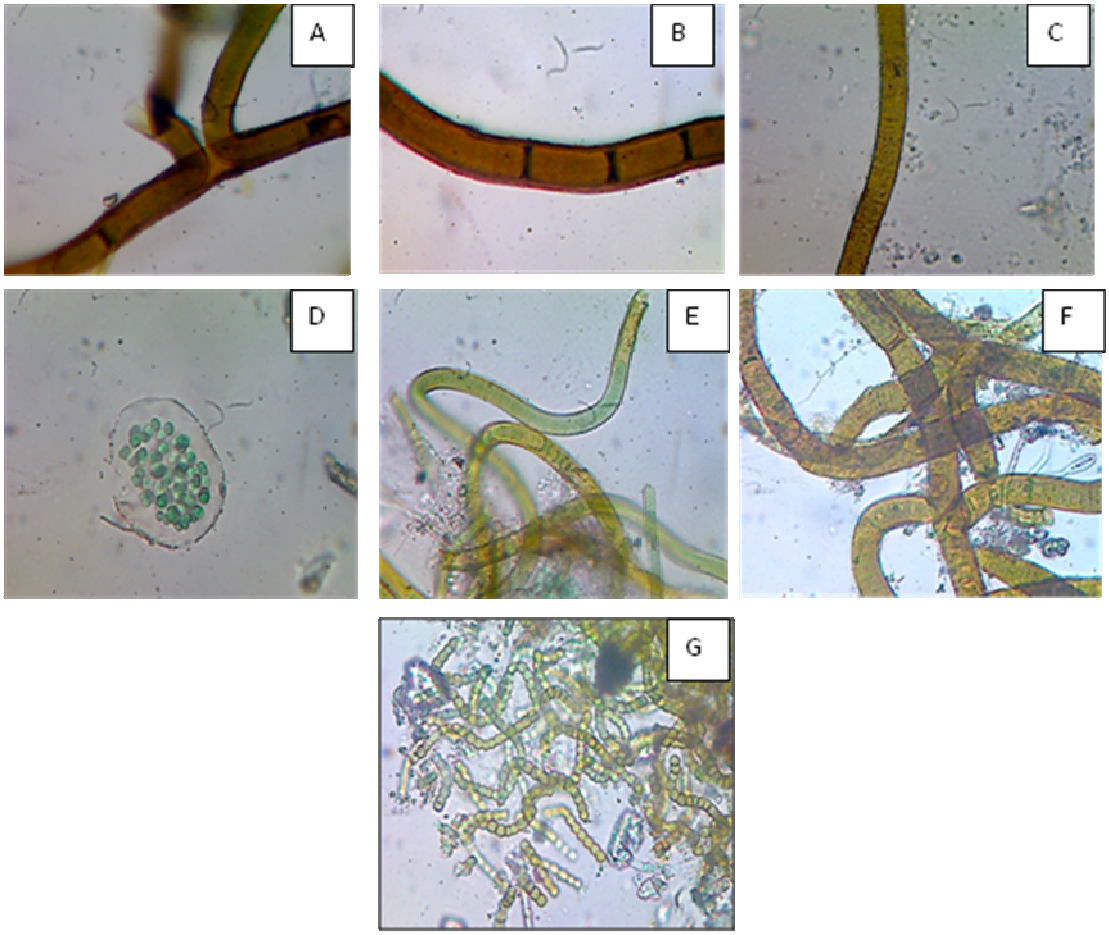

Fig. 2: Microphotographs of cyanobacteria isolated from habitats having harsh environmental conditions. (A) *Scytonema geitleri* from roof top, (B) *Scytonema* sp. having photoprotective pigment scytonemin in its outer sheath, (C) *Lyngbya arboricola* from bark of mango trees, (D and E) *Nostoc* sp. and *Scytonema* sp. respectively from window ledge, (F) *Scytonema* sp. from agriculture field and (G) *Scytonema* sp. from usar land

Cyanobacteria namely *Scytonema* sp., *Lyngbya* sp. and *Nostoc* sp. were the major taxa in most of the samples (Table 1). The exposed roof top was covered by dark brown coloured crust composed mainly of heterocystous and filamentous cyanobacterium *Scytonema geitleri* whereas the bark of mango tree (*Mangifera indica*) primarily composed of the non heterocystous, filamentous and non-spore forming cyanobacterium *Lyngbya arboricola*. *Scytonema* sp. was prominent taxa, present in the crusts collected from usar land and window ledge whereas agriculture field harboured a mixture of cyanobacterial species (Fig. 2).

Table 1: Cyanobacteria inhabiting stressed habitats
+ Present, - Absent

Organisms	Habitats				
	Window ledge	Usar land	Roof top	Agriculture field	Bark of *Mangifera indica*
Lyngbya arboricola	-	-	-	-	+
Lyngbya sp.	-	+	-	+	-
Nostoc commune	-	-	-	+	-
Nostoc sp.	+	-	-	+	-
Anabaena sp.	+	-	-	+	-
Scytonema geitleri	-	-	+	-	-
Scytonema sp.	+	+	-	+	-

3.2. Pigment profile

Pigment profile of biological crusts from different habitats is given in Fig. 3. The absorption spectra of 90 % (v/v) methanolic extract showed absorption at 665 nm due to chlorophyll *a*, at 470 nm due to carotenoids, at 309-362 nm due to MAAs and at 386, 278 and 254 due to scytonemin.

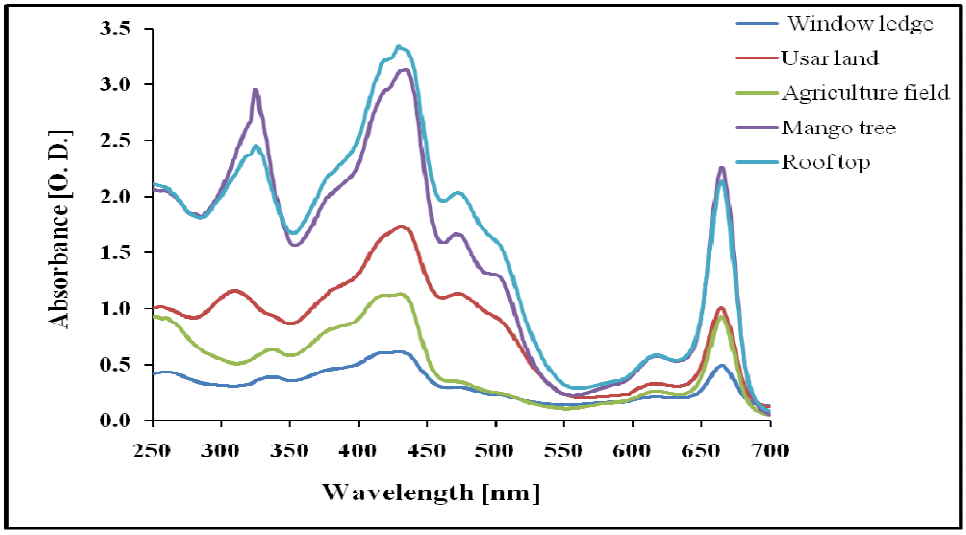

Fig. 3: Absorption spectra of methanolic extracts of cyanobacteria harboring various stressed habitats

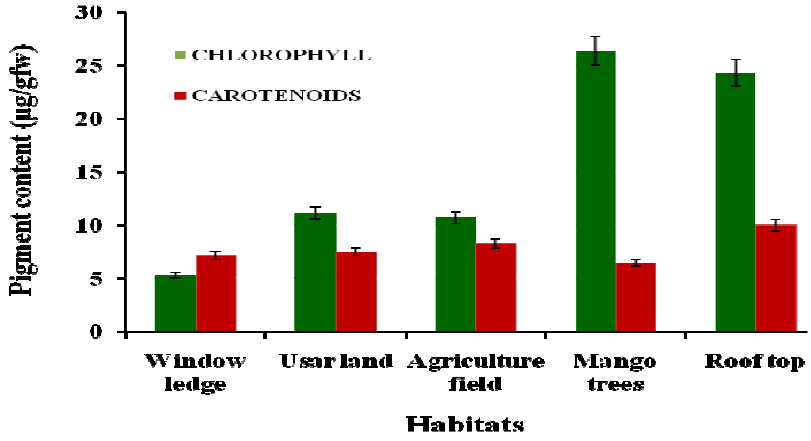

Fig. 4: Pigment analysis of cyanobacterial samples collected from habitats having harsh environmental conditions

In all the crust samples scytontemin content (mg/gfw) and MAAs concentration was found to be quite prominent, indicating their vital role in survival of organisms such as cyanobacteria in extreme environmental conditions in addition to photoprotection. Chlorophyll and carotenoid content of biological crusts collected from different habitats has been shown in Fig. 4

3.3 HPLC analysis and partial purification of scytonemin and MAAs

In the present study we made an attempt to partially purify scytonemin and MAAs from the biological crusts. HPLC chromatograms of partially purified aqueous solution from different biological crusts reveal the presence of MAAs in all of the samples and have a strong absorbance in the ultraviolet region of the spectrum with major peaks with absorption maxima at 309, 330 and 332 nm (Fig. 5).

Scytonemin was found to be present in all of the samples and has a strong absorbance in the ultraviolet region of the spectrum with major peaks in the UV-A region (at 386 nm) and continue up to UV-C region (at 254 nm) (Fig.6). Scytonemin content was found to be highest in the crust collected from roof top and minimum in the crust collected from window ledge, BHU (Fig. 7). MAAs was found to be present in all samples with maximum concentration in the crust collected from agriculture field and minimum in the crust collected from mango tree, BHU (Fig. 8). The HPLC purified fraction of MAAs from *Scytonema geitleri* and *Lyngbya arboricola* collected from roof top and bark of mango tree showed maximum absorption at 309 nm (mycosporine-taurine) and 310 nm (mycosporine-glycine) respectively. Photoprotective compounds extracted and partially purified from different environments with their corresponding absorption maxima and retention times have been listed in Table 2.

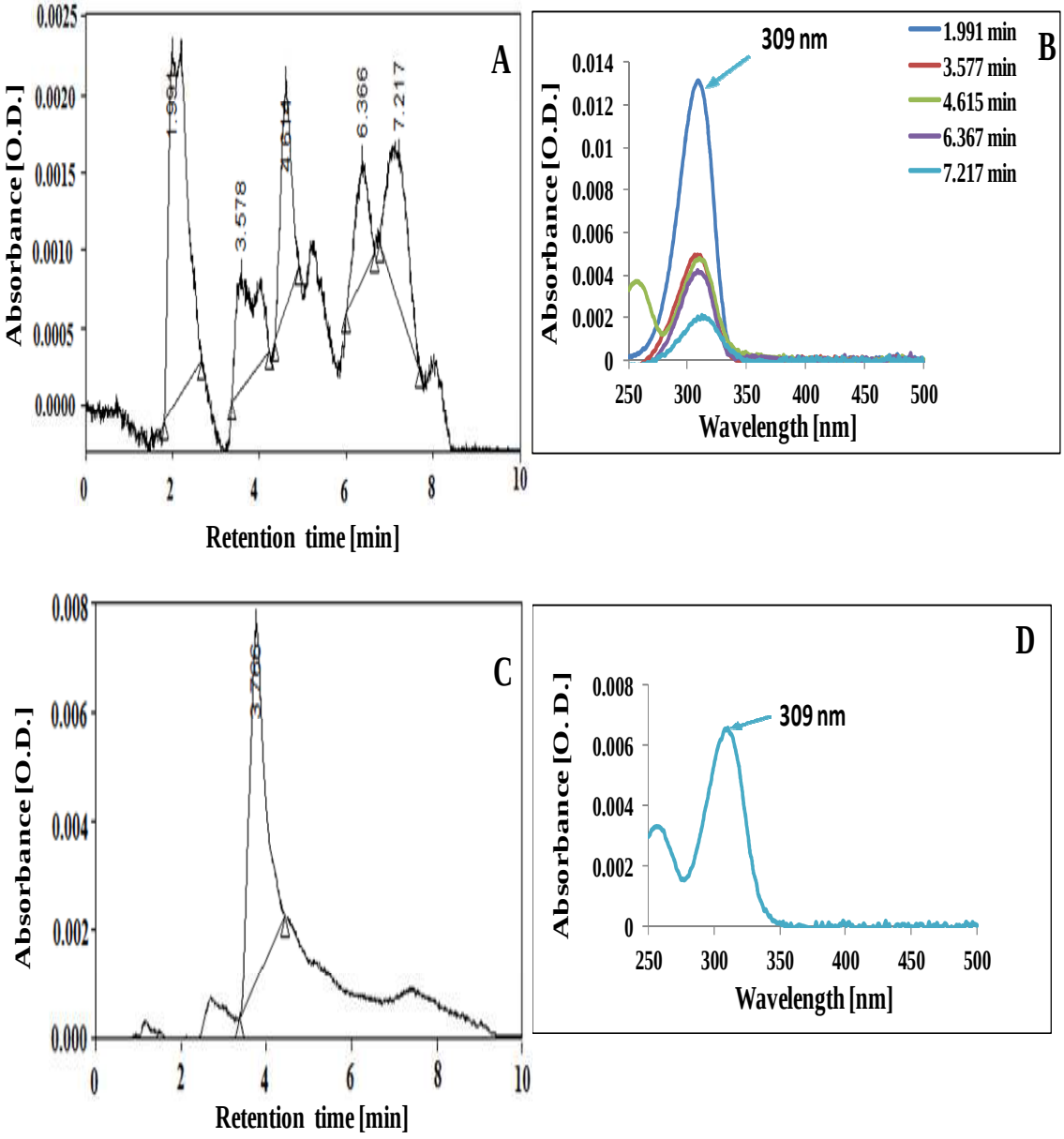

Fig. 5: HPLC chromatograms of partially purified MAAs and its corresponding absorption spectra from cyanobacterial samples collected from Roof top (A, B) and bark of *Mangifera indica* (C, D)

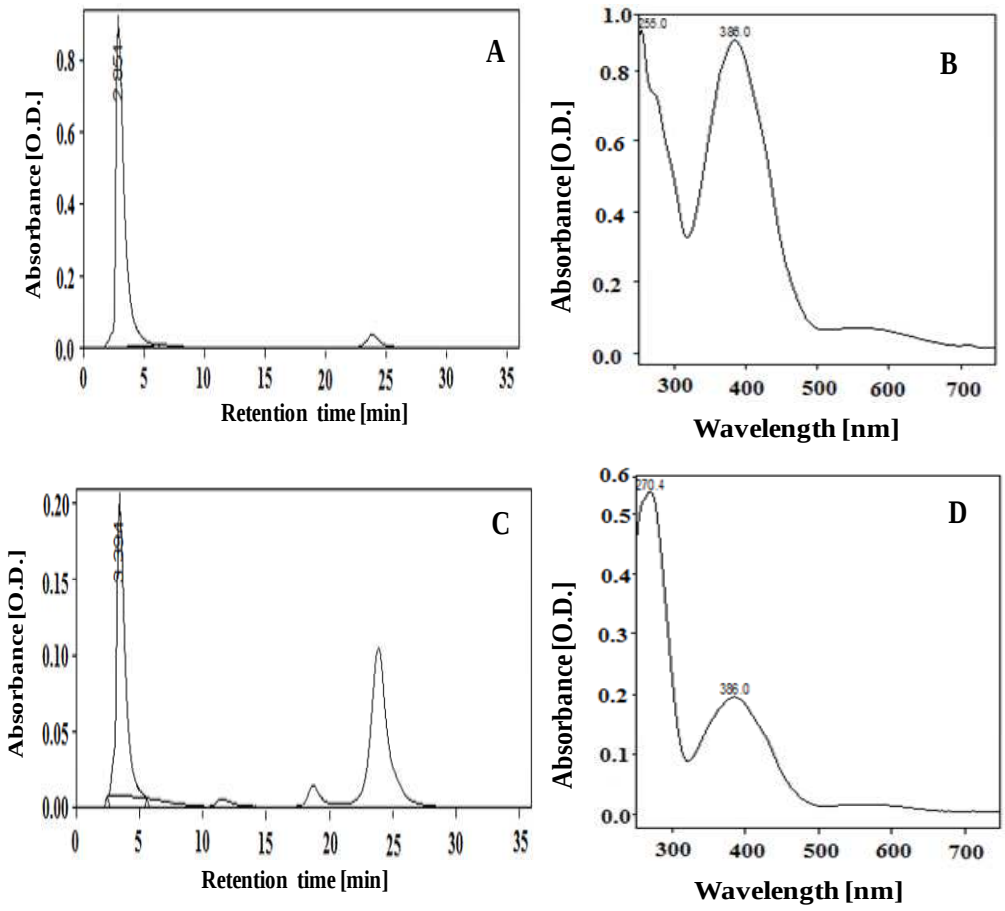

Fig. 6: HPLC chromatograms of partially purified scytonemin and its corresponding absorption spectra from cyanobacterial samples collected from Roof top(A, B) and bark of *Mangifera indica* (C, D)

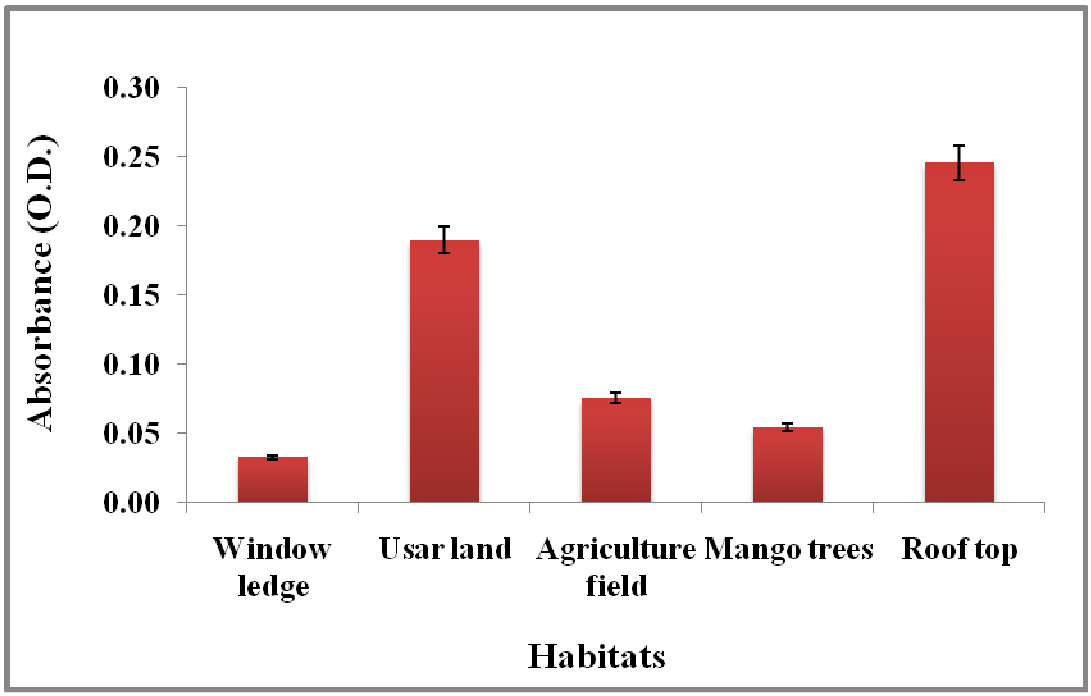

Fig. 7: Scytonemin content of cyanobacterial samples collected from various stressed habitats

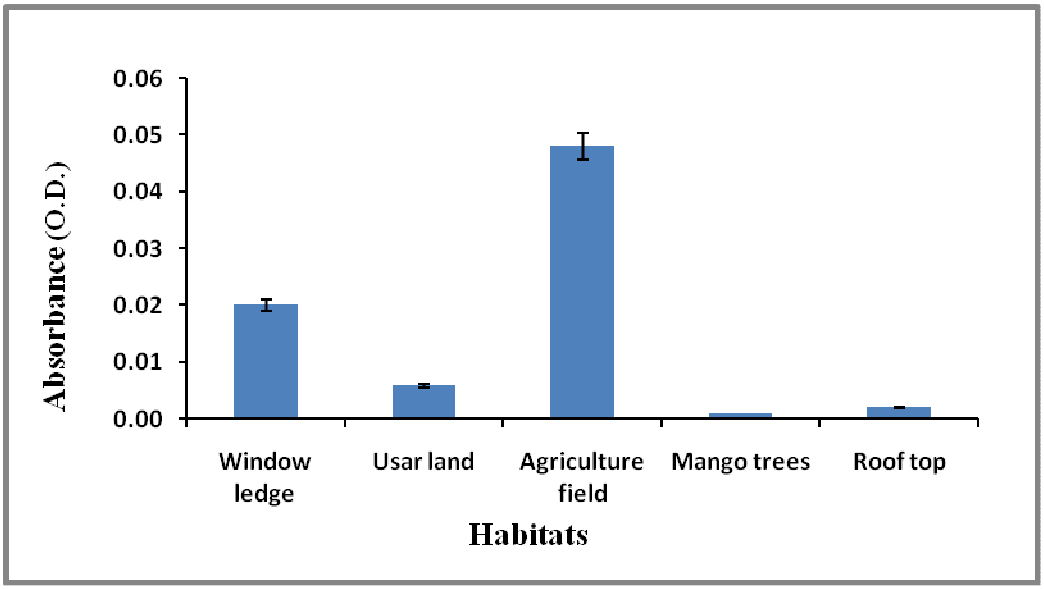

Fig. 8: MAAs concentration (based on relative absorbance of peaks) extracted from cyanobacterial samples inhabiting stressed habitats

Table 2: Photoprotective compounds from various stressed habitats with their corresponding absorption maxima and retention times

Habitats	Photoprotective compounds				
	SCYTONEMIN		MYCOSPORINE-LIKE AMINO ACIDS (MAAs)		
	λ_{max} (nm)	Retention Time (min)	λ_{max} (nm)	Retention Time (min)	MAAs Present
Roof Top	253.8, 386.0	2.85 3.39	308.0, 309.0	1.99, 3.55, 4.61, 6.36, 7.21, 4.48	Mycosporine-taurine, Mycosporine glycine
Bark of *Mangifera indica*	270.4, 384.8	3.39	309.0	3.76	Mycosporine glycine
Window ledge	255.0, 278.7, 383.5	3.49	332.0	2.96	Palythinol
Usar land	384.8	3.83	309	3.81	Mycosporine glycine
Agriculture field	383.5	1.43	329.9	2.95	Asterina-330

DISCUSSION

Cyanobacteria are possibly the most ancient photosynthetic microorganisms that have several mechanisms including synthesis of UV-absorbing compounds like MAAs and scytonemin, to mitigate the consequences of UV radiation in their natural habitats. We have selected five different habitats each representing a different environmental condition. Roof top represents a habitat having high solar irradiance, high desiccation, high temperature and lack of organic nutrients. Cyanobacteria inhabiting bark of *Mangifera indica* faces high desiccation but low solar irradiances. Usar land represents a habitat facing nutrient depletion conditions, high solar irradiances, high temperature and desiccation. Window ledge faces desiccation and moderate solar intensity whereas cyanobacteria inhabiting agriculture field receives moderate solar irradiances, rich nutrient supply and moisture. *Lyngbya* sp. and *Scytonema* sp were the dominant cyanobacterial taxa in most of the crusts. In the present study, the UV-absorbing compounds mainly scytonemin and MAAs from biological crusts were screened and partially purified through HPLC. Though the temperature in some of these habitats goes beyond 45˚C, along with high light intensity UV, extreme dryness and nutrient depleted conditions, cyanobacteria survive in these habitats due to effective adaptation strategies like presence of UV-absorbing compounds like scytonemin and MAAs [42]. HPLC analyses of the photoprotective pigment extracted from biological crusts exhibited the existence of an UV-absorbing compound, scytonemin, with absorption maxima at 386 nm and MAAs with absorption maxima at 310, 330 and 332 nm. Synthesis of scytonemin was found to be higher than carotenoid and chlorophyll in all the crust samples collected from different harsh habitats. MAAs were present in all samples. This investigation indicates that the cyanobacteria, which are predominantly present in all biological crust samples, can protect themselves from harmful doses of UV radiation by inducing the synthesis of scytonemin and MAAs as a cellular defense mechanism in addition to other defense strategies. Scytonemin content was highest in crust samples collected from roof top, as it faces several environmental stresses (high UV, temperature, desiccation and nutrient depleted conditions) followed by samples from usar land where nutrient depletion condition and high solar irradiances are major stress factors. Presence of scytonemin and MAAs in the cyanobacteria dominated crust samples collected from bark of mango trees reflects their vital role in desiccation stress tolerance also. Scytonemin has great potential in photoprotection and genome

maintenance by minimizing the cellular damage from UV-induced ROS and thymine dimer formation [8]. The biosynthesis of these photoprotective compounds is greatly affected by different abiotic stress [43, 44]. Synthesis of scytonemin has been shown to be enhanced by high temperature, photo-oxidative stress and periodic desiccation stress [45]. High temperature, strong illumination intensity, and light–dark cycle (12:12 h), nitrogen deficiency and salinity were found to elevate the synthesis of scytonemin [46-48]. Osmotic stress and elevated UV-A exposure in combination with temperature or photooxidative stress induces the synthesis of scytonemin [43]. UV radiation plays a key role in promoting the synthesis of scytonemin [49].

MAAs not only plays a significant role as photoprotective compounds [12] but also act as an osmoregulator in certain cyanobacteria, indicating that MAAs are osmotically active compounds and their accumulation in the cell is regulated by osmotic mechanism [22]. MAAs provide photoprotection against UV radiation in organisms having shikimate pathway such as arthropods, rotifers, molluscs, fishes, cnidarians, tunicates, eubacteriobionts, poriferans, nemertineans, echinodermates, platyhelminthes, polychaetes, bryozoans and protozoans. In the shikimate pathway lacking animals these compounds are derived from their algal diet [50]. Field populations of halotolerant cyanobacteria accumulate high concentration of MAAs [28]. The synthesis of MAAs in cyanobacteria is also dependent on available nitrogen [18], and growth media with a nitrogen source support the highest MAA synthesis in comparison to others without a nitrogen source. Findings from this study clearly reveals that a number of environmental factors are responsible for the synthesis of scytonemin and MAAs, but the exact mechanisms that govern the production of these photoprotective compounds are yet to be elucidated. Hence, more studies are needed to understand the induction and protection mechanisms of these ecologically important compounds in cyanobacteria, which are dominant organisms of biological crusts, under varying environmental conditions including UV radiation.

Acknowledgements
J. Pathak and V.K. Kannaujiya are thankful to CSIR, New Delhi, India for the financial support in the form of JRF and SRF respectively. This work was also partially supported by Department of Science and Technology sponsored project (No. SR/WOS-A/LS-140/2011) sanctioned to Richa. A. S. Sonker and Rajneesh are thankful to UGC and DBT for providing the fellowship in form of SRF and JRF respectively.

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