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RESEARCH ARTICLE

EFFECTS OF NITROGEN NUTRIENT ON THE PRODUCTION OF MYCOSPORINE-LIKE AMINO ACIDS IN NOSTOC SPECIES

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Abstract

Mycosporine-like amino acids MAAs belong to a family of secondary metabolites produced by a wide range of different organisms. These compounds had several potential applications in biomedical, in cosmetics and toiletries. In the present study, three Nostoc strains distributed in soil and freshwater were tested for MAA production under different nitrogen conditions. UV-B radiation significantly enhanced the production of MAAs in Nostoc linckia, Nostoc muscorum and Nostoc paludosum under nitrogen sufficient and deficient conditions. The contents of MAAs significantly increased in these three Nostoc strains with prolonged UV-B treatment under both nitrogen conditions. Both UV-B exposed N. muscorum and N. paludosum produced much less MAAs under nitrogen deficiency in comparison to culture under nitrogen sufficiency, respectively. There was just little difference of MAA contents in UV-B treated N. linckia when it was cultured under both nitrogen conditions. After separation by HPLC and characterization by in-line absorption spectra and mass spectra, one type of MAA porphyra was induced by UV-B in N. linckia under nitrogen sufficient and deficient conditions, and N. muscorum synthesized another kind of MAA shinorine under nitrogen sufficient and deficient conditions. However, UV-B induced N. paludosum to produce porphyra under nitrogen sufficient condition but shinorine under nitrogen deficient condition. These results indicated the nutrient nitrogen levels could regulate the types and amounts of MAAs in Nostoc species. These strains would be a better source for the industrial production of MAAs that can be used as organisms for studying the biosynthetic route of MAAs in nitrogen conditions.

Introduction:-

The depletion of the stratospheric ozone layer by the release of anthropogenic atmospheric pollutants such as chlorofluorocarbons, chlorocarbons and organobromides has resulted in an increase in ultraviolet radiation (UVR)

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reaching on the Earth's surface[1,2]. UVR, especially UV-B radiation (280–320 nm) induced damage for most living organism by producing sunburn, skin cancer and aging in humans and animals, pigment and growth reductions, photosynthetic damage and protein and DNA damage in plants, algae and cyanobacteria[2,3]. Cyanobacteria, the largest group of oxygenic photoautotrophic prokaryote have evolved various mechanisms such as Mycosporine-like Amino acids (MAAs) to cope with UV radiation in their natural habitat[4].

MAAs are water-soluble substances and colorlesswith range of molecular weights from 188 to 1050 Da[5,6]. They have strong UV absorption maxima from 310 to 362 nm and high molar extinction coefficients (e= 28,100–50,000 M-1 cm-1)[5,7]. They are characterized by a cyclohexenone or cyclohexenimine chromophore conjugated with the nitrogen substituent of an amino acid or its imino alcohol[1,8,9].More than 40 kinds of structurally different MAAs or MAA derivatives have been reported in diverse organisms[8,10].They have gained considerable attention for their role in UV photoprotection to absorb the harmful UV radiation by converting it into heat without generation of ROS[11,12]. MAA have also potential application in biomedical, in cosmetics and toiletries[1,13].

Nostoc are cyanobacteria widely spread in various environments including arid, semiarid and desert environments, freshwater, paddy soils and so on[14,15,16]. They can develop heterocysts to fix atmospheric N_2 and also contributed to the improve soil fertilization[17,18]. Mycosporine-like amino acid (MAA) production is one of the mechanisms developed by Nostoc species to tolerate UV during their evolutionary history[19]. This work will evaluate the nitrogen levels on the MAA production in three Nostoc strains

Materials and Methods:-

Organism and culture conditions

Nostoc linckia FACHB 391, Nostoc muscorum FACHB 395, and Nostoc paludosum FACHB 89, were obtained from the institute of Hydrobiology, the Chinese academy of Sciences. Each strain was cultured both in nitrogen sufficient BG11 media and nitrogen deficient BG110 media, in which the nitrate was removed (Supplementary table 1). All the Nostoc strains were grown under white fluorescent lamps at intensity 30 μmol photons $m^{-2}s^{-1}$and 25 °C. The cultures were shaken three times per day. After 14 days, the culture were transferred and exposed to fluorescent UV-B lamps (TL 40W/12 RS; Philips, Germany) with intensity 0.15 $W \cdot m^{-2}$ in addition to white light illumination for 7 more days. The UV-B rays were emitted from fluorescent UV-B lamps (TL 40W/12 RS; Philips, Germany). Ultra white glass was used to filter radiation below 280 nm[20]. The biologically effective UV-B radiation was then calculated according to the biological spectral weighting function of Flint and Caldwell (2003) (fig. 1). After growth, samples were collected by centrifugation and kept at -80^{o}C until MAA characterization.

Measurement of chlorophyll fluorescence

The liquid cyanobacterial culture from the white light culture were placed into petri dishes and exposed to UV-B with intensity 0.15 $W \cdot m^{-2}$ up to for 4 h at 25 0C. TheFv/Fm values were measured by Plant Efficiency Analyser (PEA, Hansatech Instruments Ltd, King's Lynn and Norfolk, UK). Samples were dark adapted for 10 minutes before measurements. Each sample was replicated three times (n=3).

Induction of MAAs under UV-B stress.

Nostoc strains were exposed to UV-B lamps with intensities of 0.15 W·m-2 in petri dishes glass for 24 and 48 hours to induce MAA production. After UVB treatment, cells were collected by centrifugation at 6000 rpm for 5 min and MAAs were extracted in 4 mL of 100% HPLC grade methanol overnight at 4^{o}C. The methanol extracts were then centrifuged 6000 rpm 5 min, and supernatant was transferred to a 2 mL Eppendorf tube. Supernatants were scanned by UV-Visible spectrophotometer between 200 and 800 nm. Data and peaks were analyzed by Probe software.

Extraction and identification of MAAs

Samples stored at -80 °C were used for MAAs extraction by methanol. After centrifugation at 6000 rpm for 5 min the methanolic extracts were collected and dried in vacuum concentrator (Labconco, UK). The dried metabolite was re-dissolved in ultrapure water and the pigments were remove in the solution with equal volume of chloroform. The MAA were then analyzed by liquid chromatography-mass spectrometry (LC-MS) analysis on Agilent technologies 6540 UHD Accurate-Mass Q-TOF. The solution sample was injected into a reversed-phase HPLC system coupled with in-line absorption spectral scans and equipped with column Inertsil ODS-SP (5 μm, 4.6 mm × 250 mm, GL Sciences Inc, Japan).The MAAs were detected at 330 nm after separation by 1 ml·min-1 of binary gradient elution of mobile A (methanol) and mobile B (water) (0-7 min, 1%-20% mobile A; 7-9 min, 20%-50% mobile A; 9-17

min, 50%-80% mobile A; 17-22 min, 80% mobile A). The electrospray interface (ESI) source and positive mode was used for mass spectrometer .

Results:-

Photosynthetic tolerance of Nostoc strains under UV-B radiation

The Fv/Fm significantly decreased in the three cyanobacterial strains during UVB treatments. Under the nitrogen sufficient conditions, the Fv/Fm values decrease 48% in N. linckia, 62% in N. muscorum and 42% in N. paludosum after UVB treatments for 4h (Fig.2). Whereas the Fv/Fm value decrease 35% in N. linckia, and 62% in N. muscorum and N. paludosum when they were cultured in nitrogen deficient media and exposed to UVB for 4h. The decrease of Fv/Fm indicated negative effects of UV-B treatments in these three Nostoc strains (Fig.2).

Induction of MAAs in Nostoc strains

The absorption spectra at the range of 200 to 800 nm were recorded for the methanolic extracts from Nostoc strains. The peaks at 435 nm and 665 nm represented the presence of chlorophyll a. The absorbance between 300 and 360 with maximum at 330 nm typically represented the presence of MAAs. The MAA contents increased in these Nostoc strains along with prolonged UVB treatments under both nitrogen levels (Fig. 3). Enhancement of MAA production by UVB suggested protective role of MAAs in Nostoc strains. Both UV-B exposed N. muscorum and N. paludosum produced much less MAAs under nitrogen deficiency in comparison to culture under nitrogen sufficiency, respectively. There was just little difference of MAA contents in UV-B treated N. linckia when it was cultured under both nitrogen conditions.

Characterization of MAAs in Nostoc strains

The kinds of MAAs were characterized as shown in Figure 3. The HPLC profiles at detecting wavelength of 330 nm showed the same peak at 2.7 min from methanol extracts in N. linckia cultured in both nitrogen level media (Fig. 4A). The compounds corresponding to the peaks showed similar absorption spectra with peak at 334 nm. Mass spectra of these compounds further showed prominent ion peaks of protonated fragment [M+H] at m/z 347.1451 from the replete nitrogen culture and 347.1447 from deficient nitrogen culture, suggesting the molecular weight of 346 for both compounds (Fig. 4A). The absorption spectra and mass spectra were consistent with the characteristics of previously recognized MAA as porphyra. According to the same analysis, N. muscorum produced the same kinds of MAA shinorine under both different nitrogen levels conditions (Fig. 4B). However, UV-B induced N. paludosum to produce porphyra under nitrogen sufficient condition but shinorine under nitrogen deficient condition (Fig. 4C).

Discussion:-

Nostoc species are widespread over the globe. They have been found in freshwater as free-living colonies or attached to rocks or at the bottom of lakes, soils, and both extremely cold and extremely arid habitats. N. linckia was found macroscopically on soils covered by meadow-halophilous vegetation[22], in freshwater[23,24]. N. muscorum are found in saline environments[25], soils and freshwater[23]. N. paludosum was found in freshwater[24] and paddy soils[26].

Production of MAAs is the well-known mechanism in cyanobacteria to tolerate UV-B radiation[27]. In the present investigation, the three Nostoc strains provided the useful producers for the production of MAAs shinorine and porphyra. Shinorine was also produced as the single MAA in Nostoc sp. ISC26 and Nostoc punctiforme under UV radiation[28,29]. Although both Nostoc commune strains from rice fields produced shinorine under UV-B radiation[17,30], Nostoc sp HKAR-2 and Nostoc sp HKAR-6 isolated respectively from the hot springs of Rajgir and rice paddy fields were found to produce both shinorine and porphyra[31]. Furthermore, the Nostoc commune strain of KU002 from Kakuma campus of Kanazawa University produced porphyra-334 and several glycosylated porphyra-334 derivatives, while another strain of KU006 from the same place biosynthesized glycosylated mycosporine-2-(4-deoxygadusolyl-ornithine)[6,32]. Recently, the terrestrial cyanobacteria Nostoc flagelliforme produced mycosporine-2-(4-deoxygadusolyl-ornithine), and an aquatic cyanobacterium Nostoc verrucosum produced shinorine and porphyra[6,32]. It seemed that the kinds of MAAs varied a lot in Nostoc species. The Nostoc strains would be prolific sources for MAA production.

Nitrogen limitation decreases the quantity of MAA production[34] whereas high concentrations of ammonium significantly promote their accumulation[1]. In the present study, the content of MAA in absence or in presence of nitrogen significantly increases by UVB-treatment in N. linckia, N. muscorum and N. paludosum. However, there was just little difference of MAA contents in UV-B treated N. linckia when it was cultured under both nitrogen

conditions, although UV-B exposed N. muscorum and N. paludosum produced much less MAAs under nitrogen deficiency in comparison to culture under nitrogen sufficiency, respectively (Fig. 3). MAAs are the nitrogen rich compounds and require nitrogen for their synthesis. Early investigations suggested nitrogen could act as a limiting factor in MAAs biosynthesis and addition of nitrogen to the growth medium enhanced their biosynthesis[35] (Singh et al., 2008). In the cyanobacterium Anabaena variabilis, the increase in quantity of MAAs synthesis was dependent on available ammonium combined with UV stresses[35]. Thus, nitrogen levels not only affected the amount of MAAs but also the qualitative bioconversion of a primary MAA to a secondary MAA[36].

The biochemical pathway has been established for shinorine synthesis. Desmethyl-4-deoxygadusol synthase and O-methyltransferase catalyze sedoheptulose-7-phosphate to form 4-DG, and ATP-grasp ligase exclusively ligated glycine moiety into 4-DG to produce mycosporine-glycine[29,37]. Shinorine was then yielded through condensing serine with mycosporine-glycine by non-ribosomal peptide synthase-like enzyme or by D-ala D-ala ligase[29,37,38]. From the structural relationship between shinorine and porphyra, it was the threonine instead of serine that was condensed to mycosporine-glycine to yield another MAA porphyra in N. paludosum under nitrogen sufficient condition[33,38]. It was likely nitrogen starvation increased the ratio of serine concentration to threonine concentration resulted in predominant shinorine formation in N. paludosum. More, in several cyanobacteria, it was observed that an addition of nitrate increase the concentration of threonine[39]. However, MAA kinds did not changed in N. linckia and N. muscorum when they were cultured under both different nitrogen levels. So regulation of MAA production by nutrient nitrogen levels was dependent on the Nostoc specific strains.

Figure 1:- Energy emission spectrum of the UV-B lamp (A), transmission spectrum of the ultra white glass filter (B), and weighted UV radiation (C) based on the biological spectral weighted function of Flint and Caldwell (2003) for UV-B treated samples.

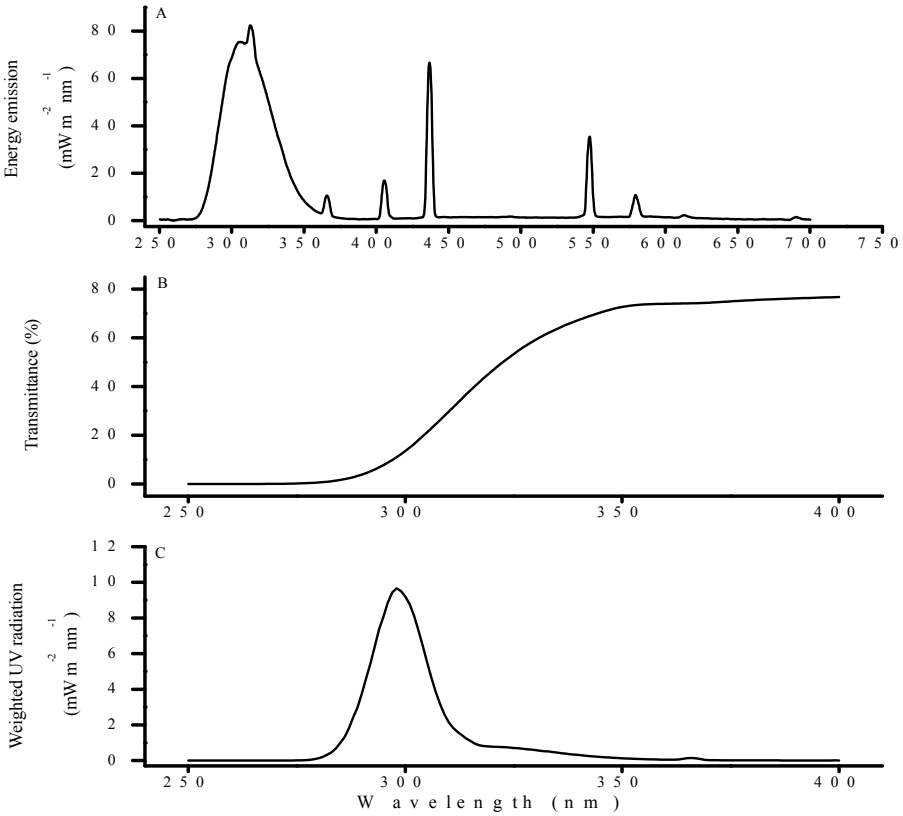


Figure 2:- Photosynthetic tolerance of the three Nostoc strains after UV-B treatments at 0.15 $W \cdot m^{-2}$ for several hours (h). The solid lines represent presence of exogenous nitrogen in the culture and the dash lines indicate absence of exogenous nitrogen in the media. There are three replicates for each treatment.

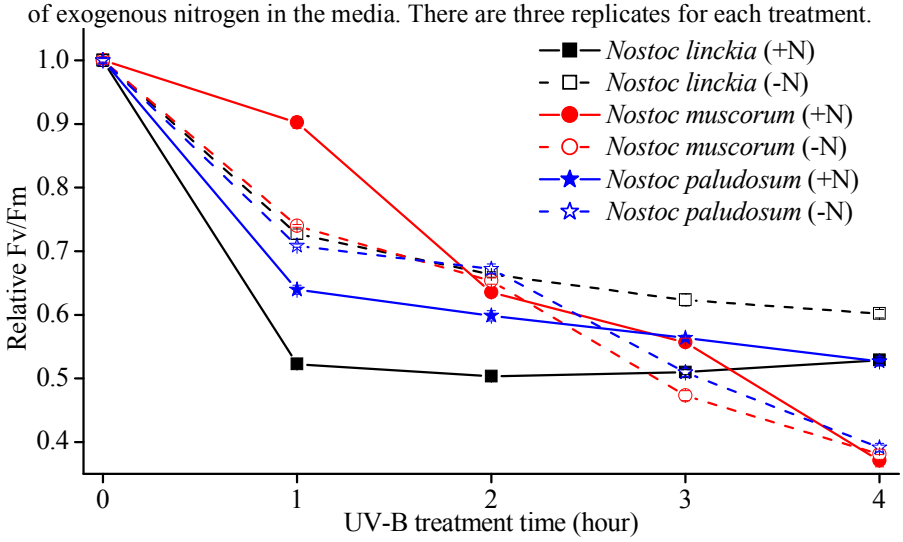


Figure 3:- Time-course of MAA induction in Nostoc strains under 0.15 $W \cdot m^{-2}$ UV-B treatments. The difference of MAA content was shown by normalized absorbance spectra of methanol extracts at a chlorophyll a peak. The solid lines represent cyanobacterial culture from normal nitrogen BG_{11} media (A-C). The dash lines indicate cyanobacterial culture from the nitrogen depleted BG_{11} media (D-F).

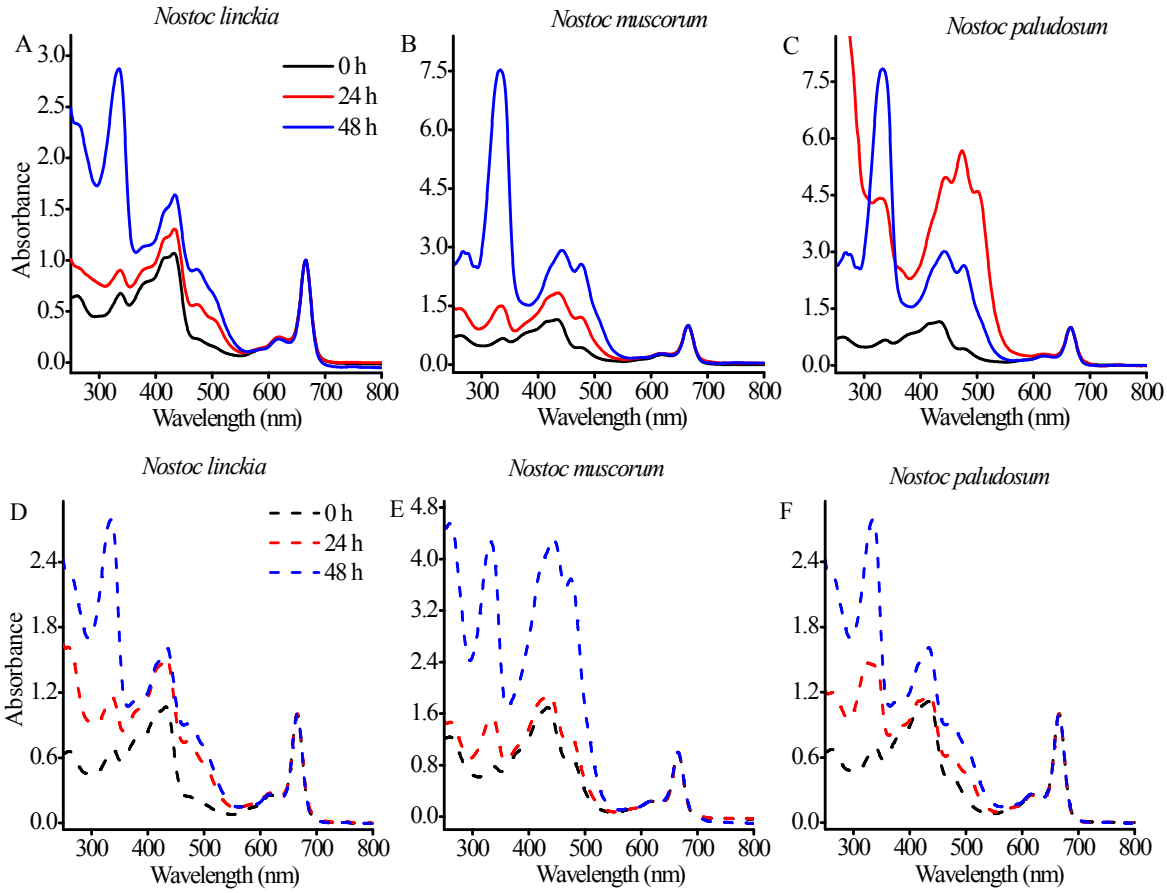
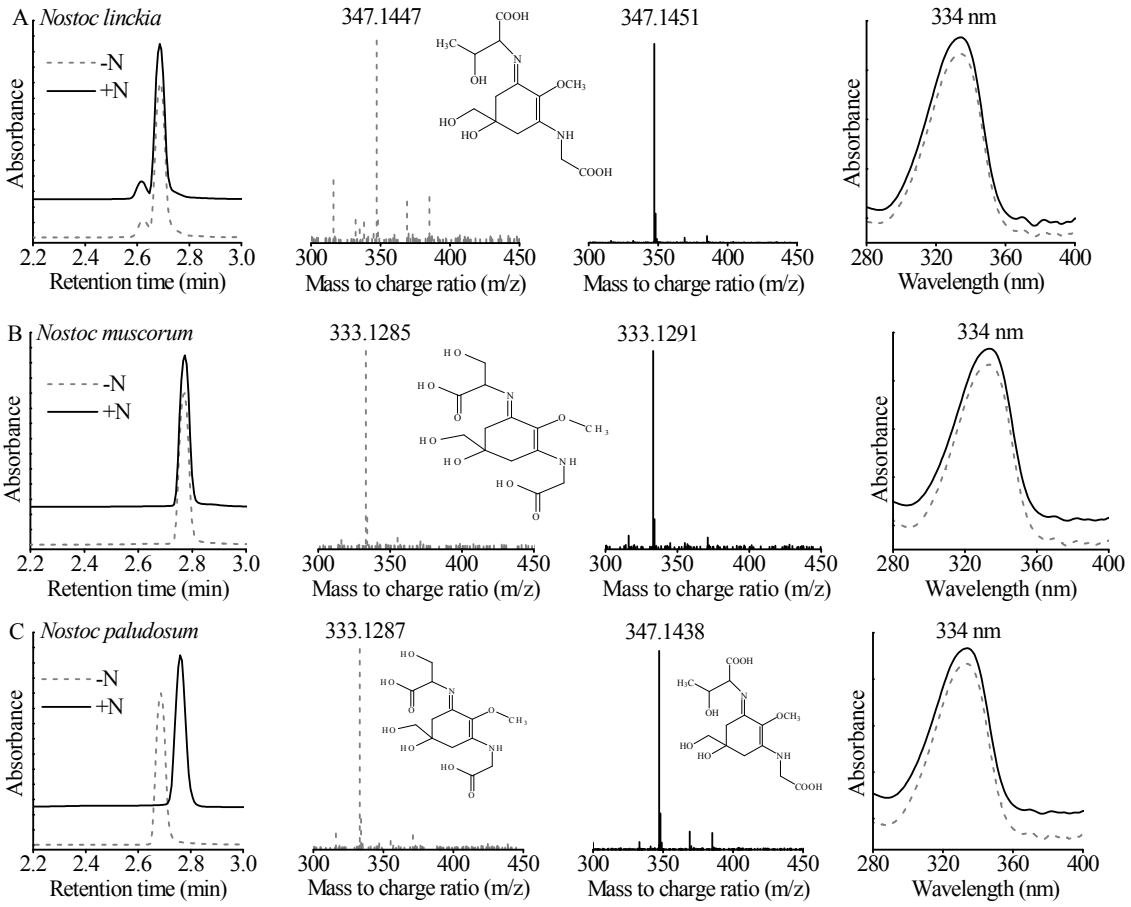


Figure 4:- MAA Characterization in the Nostoc strains from the normal BG_{11} cultures (solid lines) and nitrogen depleted BG_{11} cultures (dash lines), respectively. LC-MS analysis and in-line absorbance spectra of methanol extracts from Nostoc linckia (A), Nostoc muscorum (B), and Nostoc paludosum (C). The LC profiles were normalized by the maximum absorption of each MAA.



Supplementary table 1 Composition of BG11 Media

Reagents	BG11(+N)	BG11(-N)
$NaNO_3$	1.5 g	
$MgSO_4.7H_2O$	0.075 g	0.075 g
Na_2CO_3	0.02 g	0.02 g
K_2HPO_4	0.04 g	0.04 g
$CaCl_2.2H_2O$	0.0027 g	0.0027 g
Ferric ammonium citrate	0.006 g	0.006 g
Citric acid	0.006 g	0.006 g
$EDTANa_2$	0.001 g	0.001 g
Micronutrients	1.0 mL	1.0 mL
Distilled water	1.0 L	1.0 L
pH	7.1	7.1
Micronutrients stock solution		
H_3BO_3	2.86 g	2.86 g
9ZnSO4.7H2O	0.22 g	0.22 g
$CuSO_4.5H_2O$	0.79 g	0.79 g
$CoCl_2$	0.049 g	0.049 g
$MnCl_2.4H_2O$	1.81 g	1.81 g

$Na_2MoO_4.2H_20$	0.39 g	0.39 g
Distilled water	1.0 L	1.0 L

Conclusion:-

The kinds of MAA production varied in Nostoc strains. The nutrient nitrogen levels could regulate the types and amounts of MAAs in Nostoc species dependent manners. These Nostoc species could be used as model organisms for studying the biosynthetic route of MAA in cyanobacteria under Nitrogen levels conditions and could also biotechnologically exploited in the pharmaceutical and UV-protecting cosmetic industries.

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