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Assessing the role of temperature as an elicitor for indole-3-acetic acid production in cyanobacterial species

Priyanka Behera[1], Dhanesh Kumar[2,3], Shubhashree Mahalik[1]*

[1]P.G. Department of Biosciences and Biotechnology, Fakir Mohan University, Balasore, Odisha, India.
[2]Centre of Excellence for Bioresource Management and Energy Conservation Material Development (Under Odisha Higher Education Programme for Excellence & Equity (OHEPEE) Assisted by World Bank, Fakir Mohan University, Balasore-756089, Odisha, India.
[3]State Pollution Control Board, Regional Office, Balasore-756019, Odisha, India

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ABSTRACT

Cyanobacteria are well known for tolerance toward high level of environmental stresses. They produce several bioactive molecules as a protective measure for survival and growth under abiotic stress. The present study investigates the effect of temperature on cyanobacteria's ability to produce indole-3-acetic acid (IAA) with tryptophan as a precursor. Three cyanobacterial species, namely, *Westiellopsis* sp. TPR-29, *Hapalosiphon* sp. Ryu2-7DN_D3, and *Chlorogloeopsis fritschii* PCC 6912, were exposed to 15°C, 25°C, 35°C, and 45°C, and its effect on indole-3-acetic production capacity was tested. After 15 days incubation in above temperature, growth (as measured by cell density and chlorophyll A content); biochemical parameters such as carbohydrate, protein, lipids, and extracellular polysaccharide; and stress indicators such as malondialdehyde (MDA), catalase (CAT), and reactive oxygen species (ROS) were examined. Estimates were also made for the production of IAA. Exposure to higher temperature resulted in reduction of growth and macromolecular contents whereas ROS and MDA content increased significantly at 45°C with a concomitant increase in antioxidant enzymes like CAT. Most importantly the IAA content was observed to be higher in non-ambient conditions (15°C, 35°C, and 45°C). Production of IAA at non-ambient temperature indicates that abiotic stress like temperature variations induces phytohormone production in cyanobacterial strains as a defense strategy to protect itself against changing environmental conditions.

ARTICLE HIGHLIGHTS

Environmental stress induces phytohormones synthesis in soil microorganisms. The present work is an experimental evidence of the temperature-induced synthesis of phytohormones Indole-3-acetic acid (IAA) in filamentous and single-celled cyanobacteria. Effect of ambient (25°C) and non-ambient temperatures (15°C, 35°C, and 45°C) on IAA production was studied. The results indicate that at non-ambient temperatures, the amount of stress generated are directly proportional to amount of IAA produced.

1. INTRODUCTION

The cyanobacteria are the microorganisms that are best suited to a variety of harsh environments. The earliest known photosynthetic species are cyanobacteria, which initially appeared 3.5 billion years ago. In addition to terrestrial, freshwater, and marine ecosystems, ice shelves, bare rocks, hot springs, and arctic and antarctic lakes, cyanobacteria can be found in a variety of situations [1]. Temperature stress is one evident stress brought on by seasonal and daily variations in climate [2]. Climate change and global warming increase the importance of temperature as one of the major stress factors that could be examined to understand the survival strategy of various extremophiles [3,4]. Based on their tolerance to temperature, cyanobacteria are classified into thermophilic, mesophilic, and psychrophilic. Hence, cyanobacteria are utilized as model organisms for determining defense strategies against stresses [5]. Defense strategies depend on how the species react to and adapt to heat stress. Cyanobacteria encounter both short- and long-term stress episodes, and they can adapt to the stress by changing their morphology, metabolism, and genetic makeup [6,7].

Many agricultural soils are home to cyanobacteria, which help with biological nitrogen fixation, phosphate solubilization, and mineral release to increase soil fertility and crop yield [8-10]. However, many cyanobacteria are also known to release a variety of biologically active substances, such as proteins, vitamins, carbohydrates, amino acids, polysaccharides, and phytohormones, which act as elicitor molecules to promote plant growth and aid them in the defense against biotic and abiotic stress [11,12]. This is in addition to naturally fertilizing and balancing the mineral nutrition in the soil. Auxins, gibberellins, cytokinins, and ethylene, which are involved in plants' growth and development, are known to be significantly accumulated and released by various genera of cyanobacteria and algae [13-15]. Indole-3-acetic acid (IAA), a well-known

*Corresponding Author:
Shubhashree Mahalik,
P.G. Department of Biosciences and Biotechnology, Fakir Mohan University, Balasore - 756 089, Odisha, India.
E-mail: shubhashreemahalik@gmail.com

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auxin promotes plant development by controlling root elongation, tropic response, cell division, elongation, and differentiation [16]. IAA production has been demonstrated in a variety of cyanobacteria [17-19]. IAA-containing cyanobacterial extracts cause rooting and shooting in explants [20]. It has been noted that cyanobacteria can produce IAA by both tryptophan-dependent and tryptophan-independent pathways [21]. The following three IAA production pathways used by microbes are: Indole-3-acetamide (IAM) pathway, indole-3-acetonitrile (IAN) pathway, and indole-3-pyruvic acid (IPA) pathway. Bacteria use the IAM and IPA pathways more frequently than the IAN pathway, and they are all tryptophan-dependent [22]. L-tryptophan, a precursor to IAA, is needed by a number of cyanobacterial strains to produce the phytohormone. Cyanobacteria are a significant part of the flora in agricultural fields, thus it is crucial to understand how they contribute to the generation of IAA under temperature stress. In addition, stress alters physiological, biochemical, and morphological responses and negatively impacts cell growth and development [23].

We therefore set out to screen IAA-producing cyanobacterial strains and examine how temperature affects growth (as measured by cell density and chlorophyll A content), biochemical parameters (as measured by carbohydrate, protein, lipids, and extracellular polysaccharide), and stress markers such as malondialdehyde (MDA), Catalase (CAT), and ROS. As a result, their prospective use as a biostimulant would be established by the measurement of IAA produced by these cyanobacterial species under temperature stress.

2. MATERIALS AND METHODS

2.1. Sample Collection, Isolation, and Purification

Cyanobacterial samples were isolated from soil crust of coastal regions of Balasore district, Odisha. Crusts were inoculated on petri plates with BG-11 agar media and incubated under fluorescent light of 7.5 W/m^2 intensity. After 7 days of incubation, some cyanobacterial colonies were seen growing in the media plate. These cyanobacterial colonies were purified by repeated culturing and transfer to a new media plate. A total of seven cyanobacterial strains were isolated following this method. Subsequently, the purified strains were transferred to Erlenmeyer flasks containing 25 mL of the same medium under the same conditions for maintenance. Axenic cyanobacterial cultures were obtained by treatment with an antibiotic mixture of 200 µg/mL ampicillin and 100 µg/mL streptomycin.

2.2. Detection of IAA

Salkowski colorimetric method was used to screen IAA-producing cyanobacteria [24]. 100mg of pure cultures of cyanobacterial isolates were inoculated into BG-11 medium and incubated at RT for 14 days. The cultures were kept in an aseptic environment and supplemented with various concentrations of filter sterilized tryptophan (0.1, 0.25, 0.5, 1, 2.5, and 5mg/ml) followed by incubation under 12:12 h light: Dark cycle conditions. After 14 days of incubation, the cultures were centrifuged at 10,000 RPM for 10 min and the supernatant was collected for IAA assay. The supernatant was then exposed to Salkowski's colorimetric assay and the absorbance was measured at 535 nm.

2.3. Ultraviolet-Visible (UV-Vis) Spectrum of IAA Produced by Cyanobacteria

The spectral analysis of cell-free supernatant was done at variable wavelength using a UV-Vis spectrophotometer. The spectrum was run from 190 nm to 600 nm using appropriately diluted sample and compared with the standard IAA (11 µg/ml and 33 µg/ml).

2.4. Polyphasic Identification of Cyanobacterial Strains

Three cyanobacterial isolates that showed maximum IAA production were identified based on morphology following Komárek [25] and further confirmed by 16S rRNA gene sequencing. For phylogenetic analysis, total DNA was isolated using phenol-chloroform method. A part of the 16S rRNA gene was amplified using cyanobacterial universal primer 106F (5'CGGACGGGTGAGTAACGCGTGA3') and 1387R (5'TAACGACTTCGGGCGTGACC3'). 20 µL reaction mixture was prepared which comprises 10 µL of SRL Taq Mix polymerase chain reaction (PCR) 2X Master Mix, 2.5 µL of the template DNA, 1 µL each of 10 mM of forward and reverse primers, and 5.5 µL of sterile water. The PCR thermal cycle comprised an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min 30 s, 72°C for 1 min 30 s, and the final extension at 72°C for 15 min. The 1281 bp amplified product was purified using Qiagen Gel purification kit. The gel-purified PCR products were submitted to Eurofins Genomics India Pvt. Ltd, Bengaluru, India, for sequencing. Sequences of 16S rRNA gene were compared with NCBI sequence database (GenBank) through BLAST (www.ncbi.nlm.nih.gov/BLAST).

2.5. Exposure of Cyanobacterial Strains to Temperature Stress

A seed culture was set up with 5 ml BG11 broth to which three axenic cyanobacterial cultures were inoculated and incubated for 3 days under fluorescent light of 7.5 W/m^2 intensity at 25 ± 2°C under 12:12 h light: Dark cycle conditions. Once the cells are in log phase, they are sub cultured into four 50 ml BG 11 broth and again incubated for 5 days same conditions till the cells are in log phase. After 5 days, the four flasks were shifted to 15°C, 25°C, 35°C, and 45°C, respectively, and incubated under fluorescent light of 7.5 W/m^2 for 12:12 h light: Dark cycle conditions. After 15 days, samples were harvested for biochemical analysis and estimation of growth.

2.6. Estimation of Growth

To estimate the total biomass, 1 ml culture was taken and homogenized. The optical density of the homogenized cultures was measured at 720 nm following which the samples were dried and the dry weight was estimated. In the case of cyanobacteria, total chlorophyll content is an indirect measurement of growth. For this, 1 ml culture is centrifuged at 10,000 rpm. To the pellet, 4 ml methanol is added and vortexed thoroughly. The mixture is incubated at 60°C water bath for 1 h. After incubation, the tubes are cooled and centrifuged at 10,000 rpm and the clear supernatant is taken for measuring OD at 665 nm.

2.7. Estimation of Macromolecular Content

The total carbohydrate content was estimated using Anthrone method [26]. Total protein content was estimated using Lowry method [27]. The total lipid was estimated using Vanillin-Phosphoric acid reagent [28]. Exopolysaccharide (EPS) estimation was done by using Anthrone reagent [29]. MDA content was estimated by protocol proposed by Heath and Packer, 1968 [30], CAT assay was done as per the protocol in the cited literature [31], and ROS estimation was done by protocol proposed by Able *et al.* [32].

3. RESULTS AND DISCUSSION

3.1. Screening of IAA Producing Strains

Seven cyanobacterial isolates were purified and named as FMU-PC1 to FMU-PC7. These isolates were tested for their ability to produce IAA using varying amounts (0.1, 0.25, 0.5, 1, 2.5, and 5 mg/ml) of

tryptophan as a precursor. Production of IAA was concentration-dependent and increased with increasing concentration of L-tryptophan in the medium [Figure 1a]. This suggests that the tryptophan-dependent route for IAA synthesis is present in all strains. Spectral analysis using UV-visible spectrometry was done as a qualitative check for the sample purity and identity. The maximum absorption wavelength (λ_{max}) of IAA obtained from the cell-free supernatant of cyanobacterial samples was compared with the λ_{max} of standard IAA. The λ_{max} of purified IAA was obtained in the range of 290–294 nm whereas that of standard IAA was obtained at 290 nm which confirmed the purity and quality of IAA produced by selected cyanobacterial species [Figure 1b].

Out of seven strains, FMU-PC5 produced the highest concentration of IAA and the lowest level was observed in FMU-PC7. Since under stress conditions, different strains behave differently by altering their growth, metabolism, and physiology, we therefore selected three strains one unicellular (FMU-PC2) and two filamentous (FMU-PC1 and FMU-PC5) cyanobacteria for further study.

3.2. Molecular Analysis of the IAA-Producing Strains

Molecular characterization by 16S rRNA confirmed that FMU-PC1 pure cultures showed 100% similarity to *Westiellopsis* sp. TPR-29 (GenBank: MT350511.1), FMU-PC5 showed 100% similarity to *Hapalosiphon* sp. Ryu2-7DN_D3 (GenBank: LC325255.1), and FMU-PC2 showed 100% similarity to *Chlorogloeopsis fritschii* PCC 6912 (GenBank: MK953013.1).

3.3. Effect of Temperature on Growth

According to estimates, global warming may cause an increase of 0.2°C every 10 years [33]. Climate change-related abiotic stress induces cyanobacteria, which are known for their remarkable adaptability to many environmental situations, to change their metabolic function [5]. Temperature variation influences a wide range of biological processes, including the physiology and metabolism of cyanobacteria. The maximum biomass formation was observed at 25°C since this is the optimal temperature for cyanobacterial growth. As an indirect indicator of growth, chlorophyll A concentration is likewise reported to be maximum at 25°C. Both biomass (dry cell weight) [Figure 2a] and chlorophyll A concentration [Figure 2b] gradually decreased when temperature rose to 35°C and 45°C. The presence of chlorophyll A shows that the cells were still alive even though the dry cell weight had decreased. It is interesting that exposure to low temperatures, that is, 15°C, had little impact on growth. Cyanobacteria are widely known for their ability to live at extremely low temperatures, even down to 20°C [34]. This may be because desaturation of fatty acids in the membrane happens at low temperatures and activates certain enzymes that increase the efficiency of transcription and translation, enabling growth and metabolism at below-ambient temperatures [5]. This trend was obtained for all three strains *Westiellopsis* sp. TPR-29, *Hapalosiphon* sp. Ryu2-7DN_D3 and *C. fritschii* PCC 6912. *Hapalosiphon* sp. Ryu2-7DN_D3 and *Westiellopsis* sp. TPR-29 were more resistant to higher temperature, whereas *C. fritschii* PCC 6912 showed better growth at 15°C.

3.4. Effect of Temperature on Carbohydrate Content

It was observed that 25°C was the ideal temperature for promoting growth and carbohydrate buildup because, at this temperature, cells' capacity to use carbon and nitrogen rises dramatically. Higher temperature influences cellular physiology and results in denaturation of vital metabolites (enzymes/proteins), which lowers CO_2 fixation, while lower temperatures during growth result in reduced electron transport [35]. As a result, the carbohydrate content was lower at 15°C and 35°C than it should have been. While at 45°C, there was a drastic fall in carbohydrate content, indicating that higher temperatures impair an organism's ability to photosynthesize [Figure 3a]. All strains showed this tendency, with the exception of *C. fritschii* PCC 6912, whose carbohydrate content was nearly the same at 15°C and 25°C, showing that this species has a wide range of tolerance for low temperatures and can live and photosynthesize even at lower temperatures.

3.5. Effect of Temperature on Protein Content

Under high-temperature stress, proteins frequently aggregate and desaturate, disrupting their transport and activities [5]. This is one of the main causes of the low protein content at 35°C and 45°C. All of the three strains showed this pattern [Figure 3b]. However, compared to the ambient temperature of 25°C, the protein concentration was considerably lower at 15°C. Low protein content may be a result of the cellular metabolism slowing down at non-ambient temperatures like 15°C, which also affects growth rate and total cell biomass, as seen in our study.

3.6. Effect of Temperature on Lipid Content

The generation of fatty acids by cyanobacteria can be drastically impacted by temperature. The lipid content of the cell often changes in response to stresses including temperature, salt, desiccation, and photoinhibition [36]. In the present study, it was observed that elevated temperature led to increase in lipid content [Figure 3c]. Maximal lipid concentration was found at 35°C, while maximal biomass formation is seen at 25°C. All of the strains' total lipid contents increased

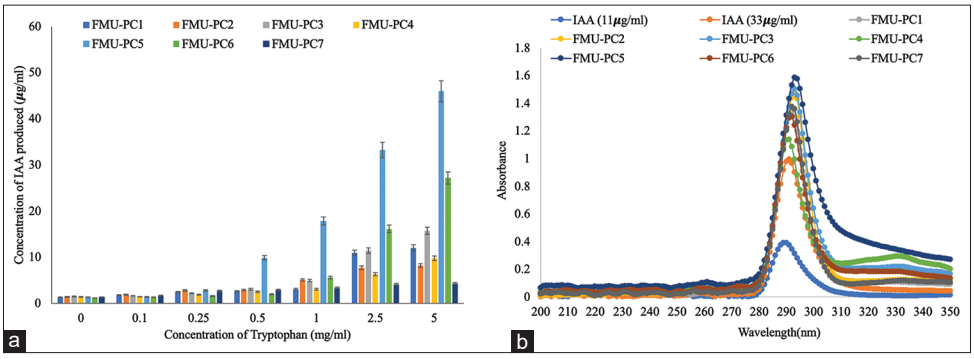

Figure 1: (a) Tryptophan concentration dependent indole-3-acetic acid production by seven cyanobacterial pure cultures obtained from coastal regions of Balasore district of Odisha, India and (b) ultraviolet-visible spectral analysis of indole-3-acetic acid of cyanobacterial samples.

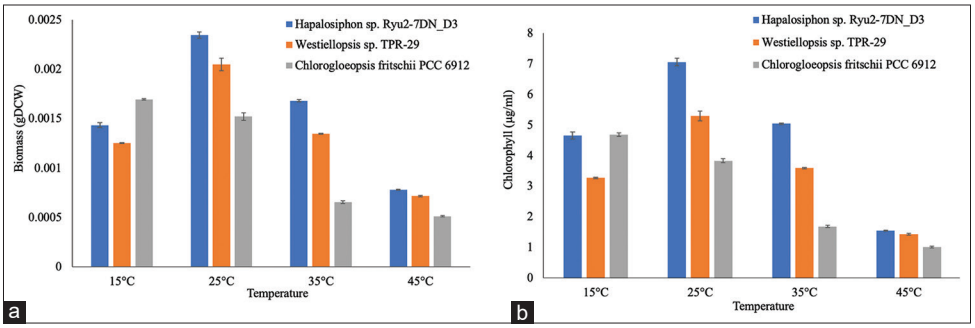

Figure 2: (a) Dry weight of cyanobacterial samples and (b) chlorophyll A content of cyanobacterial samples under different temperature.

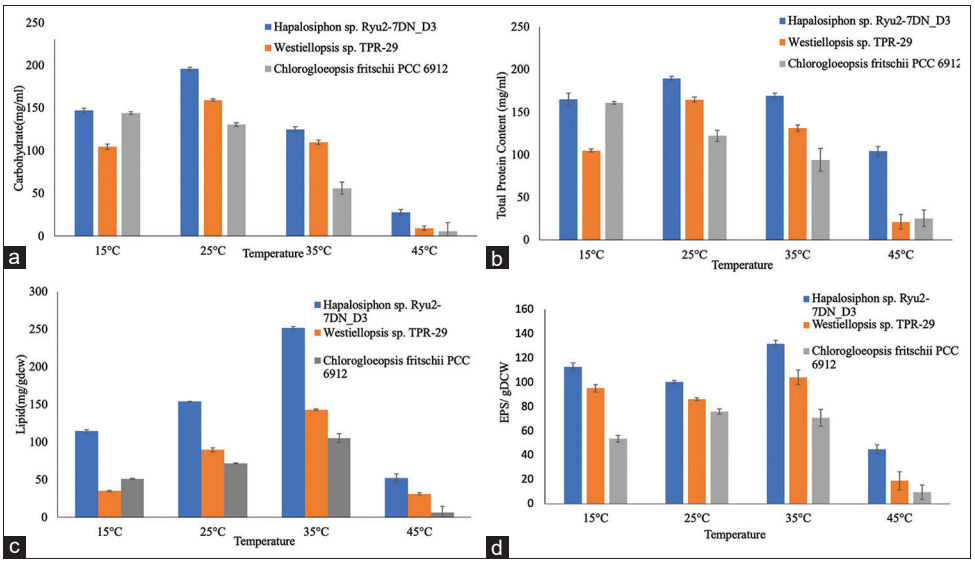

Figure 3: (a) Total carbohydrate content; (b) total protein content; (c) total lipid content; and (d) EPS content of cyanobacterial samples under different temperature.

along with the temperature as it went from 15°C to 35°C. Increased lipid production in response to temperature is likely caused by the requirement to stabilize membranes to sustain vital physiological functions. However, it was fascinating to note that very little lipid accumulation occurred at 45°C.

3.7. Effect of Temperature on Exopolysaccharide Content

EPSs provide a milieu that is structurally stable and well-hydrated for colonization of cyanobacteria. In addition, it offers chemical and physical defense against biotic and abiotic stressors. It is believed that EPS excretion serves as a physiological reaction to changes in the environment, enabling cyanobacteria to keep up their fitness while simultaneously supporting the expansion of other cohabiting organisms [37]. In this study, it was observed that the generation of EPS increased when the temperature rose from 15°C to 35°C. At 35°C, the highest EPS production was observed [Figure 3d]. According to earlier research, filamentous cyanobacteria produce their most EPS between 27°C and 34°C. Higher levels of EPS are produced and deposited at higher temperatures as a response to anticipated desiccation because desiccation is typically associated with greater temperatures [29].

3.8. Effect of Temperature on Stress Markers

Cyanobacterial physiology and metabolic behavior are predominantly impacted by abiotic stressors. As a result, a number of stimuli and other defense mechanisms are known to be induced in response to stress. One of them is reactive oxygen species (ROS) which are produced in response to various metabolic stress conditions. ROS can be reduced by a variety of enzymes, including superoxide dismutases (SOD), CAT, and guaiacol peroxidase, as well as non-enzymatic compounds such as carotenoids and glutathione reductase [38]. However, excessive free radical production can cause oxidative stress, which can harm cellular nucleic acids and structural integrity [39]. However, with an effective defense and repair system, cyanobacteria may recover from oxidative damages. This study looked at how different temperatures affected the levels of ROS [Figure 4a], CAT [Figure 4b] and MDA [Figure 4c]. In all of the non-ambient temperature conditions, as was to be predicted, the levels of all three stress markers were high, with the greatest levels being seen at 45°C [Figure 4a-c]. Antioxidant marker production suggests that non-optimal temperature causes a general stress response by generating ROS.

3.9. Effect of Temperature on IAA Production

IAA is a natural auxin that is synthesized in many species of non-seedling plants, bacteria, fungi, and algae [40]. A number of cyanobacteria have been reported to produce IAA. Several works have reported the effect of pH, salinity, incubation time, and concentration of Tryptophan on IAA production [41,42], but there are almost no records of effect of temperature on IAA synthesis by cyanobacteria. Effect of temperature on IAA production by various bacteria [43] and yeast [44] has been

Table 1: Two-way ANOVA analysis on effect of temperature on IAA production in various cyanobacterial species.

Source of variation	SS	df	MS	F	*P*-value	F crit
Cyanobacterial strains	3244089	2	1622045	47.97648	7.01E-11	3.259446
Temperature	9472744	3	3157581	93.39425	4.77E-17	2.866266
Interaction	7922317	6	1320386	39.0541	2.52E-14	2.363751
Within	1217130	36	33809.16			
Total	21856281	47				

ANOVA: Analysis of variance, IAA: Indole-3-acetic acid

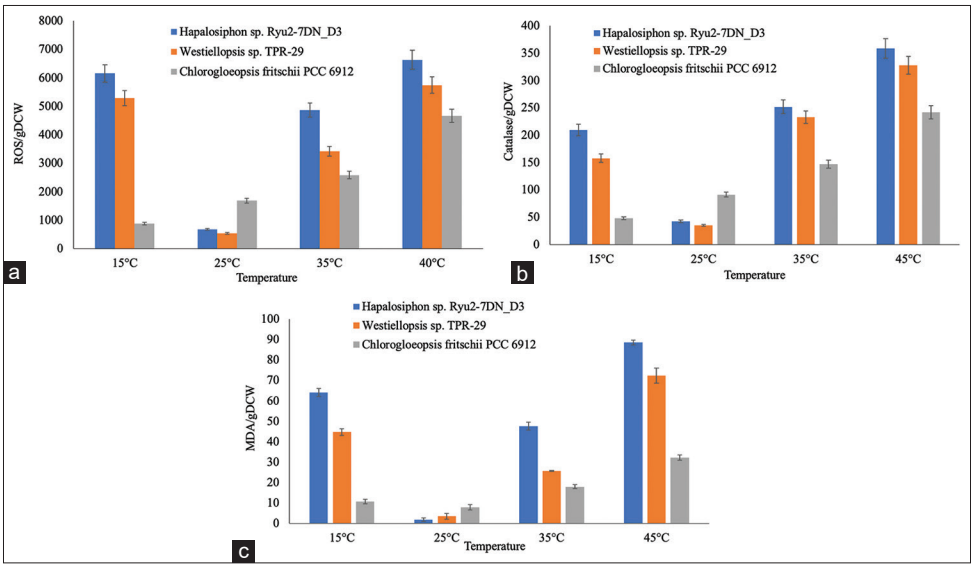

Figure 4: (a) Reactive oxygen species content; (b) catalase activity; and (c) malondialdehyde content of cyanobacterial samples under different temperature.

well established; hence, this study explored the role of temperature as an inducer of IAA production by cyanobacteria. It was observed that in all three strains IAA production was comparatively higher in non-ambient temperatures like (15°C, 35°C, and 45°C) than 25°C which is the optimum temperature for cyanobacterial growth. It was expected that at higher temperatures IAA production might be high, but in the case of *Hapalosiphon* sp. Ryu2-7DN_D3 and *Westiellopsis* sp. TPR-29, elevated amounts of IAA production were observed even at 15°C. Only *C. fritschii* PCC 6912 showed higher IAA titers at higher temperature. At 15°C, the IAA production by *C. fritschii* PCC 6912 was much less than the amount produced at optimum temperature. Overall highest titers of IAA were observed in *Hapalosiphon* sp. Ryu2-7DN_D3 followed by *Westiellopsis* sp. TPR-29 and *C. fritschii* PCC 6912 under various temperatures [Figure 5].

A two-way ANOVA was performed to analyze the effect of temperature on IAA production across different cyanobacterial strain. The amount of IAA produced by different strains at different temperature was statistically significant with a *P*-value of $2.51851e^{-14}$ ($P < 0.05$). Difference in IAA production by three cyanobacterial strains was also statistically significant with a *P*-value of $7.01295e^{-11}$ ($P < 0.05$). Similarly, statistically significant variation was observed in IAA production at various temperatures (*P*-value of $4.77249743587743e^{-17}$). The detailed two-way ANOVA analysis is presented in Table 1. This experiment indicates that any fluctuation in environmental temperature induces the cyanobacterial species to synthesize phytohormones like IAA as a defense mechanism to protect itself from metabolic stress.

Previously, it has been reported that among many compounds that cyanobacteria produce, IAA promotes growth and prevents

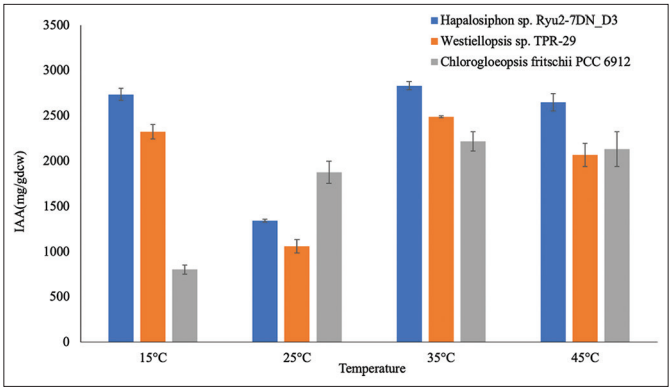

Figure 5: Indole-3-acetic acid produced by cyanobacterial samples under different temperature.

organisms from environmental stress such as desiccation, osmotic, cold shock, and heat shock. Further, IAA activates the antioxidant activity in the cell by inducing the expression of antioxidant enzymes such as CAT, SOD, and peroxidase [45]. A similar results have also been obtained in the present study which corroborates with the cited literature.

4. CONCLUSION

The present study showed that temperature stress acts as an elicitor for IAA production by cyanobacterial strains. On the basis of habitat and tolerance of cyanobacteria toward temperature, they are classified as thermophilic, mesophilic, and psychrophilic; hence, these temperature

ranges (15°C, 25°C, 35°C, and 45°C) were selected for the study [46]. As expected at optimum temperature of 25°C, the biomass and chlorophyll content as well as macromolecular content was highest for all selected species. Whereas at non-ambient temperatures, there was a concomitant decline in all growth and biochemical parameters, indicating the effect of temperature stress on physiology of the strains. As the strains were exposed to higher temperature, it resulted in reduction of growth and macromolecular contents whereas ROS and MDA content increased significantly at 45°C with a concomitant increase in antioxidant enzymes like CAT. Increase of ROS, MDA, and CAT levels at non-ambient conditions clearly emphasizes that fluctuations in incubation temperature elicit stress response in cyanobacteria. While these observations were similar across the selected temperature ranges for all three strains, but in terms of tolerance, *Hapalosiphon* sp. Ryu2-7DN_D3 was most tolerant to variations in temperature. The higher tolerance of *Hapalosiphon* sp. Ryu2-7DN_D3 is evident from the fact that it had higher growth as well as biomolecular content as compared to other strains during temperature stress. Whereas the unicellular cyanobacteria, *C. fritschii* PCC 6912 was most sensitive to temperature change. Most importantly, the IAA content was observed to be higher in non-ambient conditions (15°C, 35°C, 45°C). *Hapalosiphon* sp. Ryu2-7DN_D3 and *Westiellopsis* sp. TPR-29 produced almost similar levels of IAA at 15°C, 35°C, and 45°C. However, *C. fritschii* PCC 6912 produced IAA only at 25°C, 35°C, 45°C and the levels decreased at 15°C. Production of IAA at non-ambient temperature indicates that abiotic stress induces phytohormone production in cyanobacterial strains as a defense strategy to protect itself against changing environmental conditions.

Apart from protecting itself, cyanobacteria coexist with plants and mutually benefit each other. They not only fix nitrogen but also synthesize phytohormones like IAA that helps them in root colonization and parallelly improves plant vigor [47]. IAA isolated from yeast has been known to inhibit the growth of weeds and therefore can be used as a herbicide and replace the chemical herbicides [48]. Similarly, it has been observed that usage of IAA or cyanobacterial biofertilizer ameliorates the atrazine toxicity in paddy crops [45]. Role of cyanobacteria as biofertilizers and soil stabilizers in agriculture has been well proved where it promotes cell division and plant elongation [49]. Even they are known to protect plants from pathogenic infections [50]. Cyanobacteria has been known to increase saline tolerance in rice, where strains isolated from saline soils have led to increase in root length and promoted seedling growth and yield [51]. Gibberellic acid produced by cyanobacteria has also led to increase in shoot dry weight and carotenoid content in case of saline-stressed rice [52]. A significant contribution of cyanobacteria is the restoration of drylands in case of severe drought situations. Priming the soil with cyanobacterial strains increases water retention capacity of soils and thereby increases germination and seedling growth [53]. These characteristics of cyanobacteria are an advantage for increasing agricultural productivity. Like salinity and drought, temperature fluctuation is also an abiotic stress that inhibits plant growth, resulting in low agricultural yields. Hence, we propose that when soil temperature becomes unsuitable for plant growth, using cyanobacterial extracts as bio-stimulants may induce plant growth promotion by synthesizing various phytohormones.

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6. AUTHOR'S CONTRIBUTIONS

Conceptualization: Shubhashree Mahalik and Dhanesh Kumar; Data acquisition/analysis: Priyanka Behera and Dhanesh Kumar; Data analysis/interpretation/drafting manuscript: Shubhashree Mahalik; Critical Revision of the manuscript: Shubhashree Mahalik and Dhanesh Kumar; and Supervision and final approval: Shubhashree Mahalik.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All datasets were generated and analyzed in the present study.

11. PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

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