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Effect of temperature stress on the growth, physiological and biochemical parameters, and enzymatic and non-enzymatic activities of two *Nostoc* strains from different habitats

Efecto del estrés térmico sobre el crecimiento, parámetros fisiológicos y bioquímicos, actividades enzimáticas y no enzimáticas de dos cepas *Nostoc* de diferentes hábitats

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Abstract

There is increasing evidence regarding the influence of elevated temperatures on the growth and productivity of photosynthetic organisms. In the current research, we compared physiological and biochemical responses of *Nostoc spongiiforme* Agardh ex Bornet et Flahault (1888) (freshwater) and *Nostoc calcicola* Brébisson ex Bornet & Flahault (1886) (marine water) by batch culture under various temperatures (25-45°C). A decrease in growth and photosynthetic pigment contents was observed with rising temperature in the *N. spongiiforme,* in contrast to *N. calcicola*. Furthermore, significantly higher levels of total peroxide and hydroxyl radicals were recorded at elevated temperatures, which in turn enhanced the accumulation of *"malondialdehyde (MDA)"* and carbonyl content, indicating greater oxidative damage in *N. spongiiforme* than *N. calcicola*. An increase in proline and *"ascorbate (AsA)"* content with rising temperature suggests that the cells of both *Nostoc* spp., in an attempt to mitigate the oxidative stress induced by temperature, showed higher proline and AsA content in *N. calcicola* than

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in *N. spongiiforme.* Likewise, an increase in the activity of *superoxide dismutase (SOD)* and*catalase (CAT)* was also observed in *N. calcicola* in contrast to *N. spongiiforme*. The nitrogenease activity was also affected under different growth temperatures in both *Nostoc* spp. Hence, this study reveals that from the two *Nostoc* species studied, *N. calcicole* has the potential to thrive under changing climatic conditions. Further research will help in screening *N. calcicola* and the indentification of the genes that enable this cyanobacterium to thrive at higher temperatures, so they can be cultured in bulk and used for diverse biotechnological applications, even under extreme temperature conditions.

Keywords: Antioxidant enzyme; nitrogenase activity; oxidative damage; reactive oxygen species; temperature.

Resumen

Cada vez hay más evidencia sobre la temperatura elevada y su influencia en el crecimiento y la productividad de los organismos fotosintéticos. En esta investigación, comparamos las respuestas fisiológicas y bioquímicas de *Nostoc spongiiforme* Agardh ex Bornet et Flahault (1888) (agua dulce) y *Nostoc calcicola* Brébisson ex Bornet & Flahault (1886) (agua marina) mediante cultivo por lotes a diversas temperaturas (25-45 °C). Se observó una disminución del crecimiento y del contenido de pigmentos fotosintéticos con el aumento de la temperatura en *N. spongiiforme*, a diferencia de *N. calcicola*. Además, se registró un nivel de peróxido total y radicales hidroxilos significativamente mayores a temperatura elevada, lo que a su vez mejoró la acumulación de malondialdehído (MDA) y el contenido de carbonilo, lo que indica un mayor daño oxidativo en *N. spongiiforme* que en *N. calcicola*. Un aumento en el contenido de prolina y ascorbato (AsA) con el incremento de la temperatura sugiere que las células de ambas especies de *Nostoc*, al intentar mitigar el estrés oxidativo inducido por la temperatura, mostraron un mayor contenido de prolina y AsA en *N. calcicola* que en *N. spongiiforme*. Asimismo, se observó un ascenso en la actividad de la superóxido dismutasa (SOD) y la catalasa (CAT) en *N. calcicola*, a diferencia de *N. spongiiforme*. La actividad de la nitrogenasa también se vio afectada bajo diferentes temperaturas de crecimiento en ambas especies de *Nostoc*. Por lo tanto, este estudio revela que, entre las dos especies de *Nostoc* estudiadas, *N. calcicola* tiene el potencial de prosperar en condiciones climáticas cambiantes. Se necesitarán más investigaciones para analizar *N. calcicola* y descubrir los genes responsables de ayudar a esta cianobacteria a prosperar a temperaturas más altas, para que pueda cultivarse en grandes cantidades y usarse para diversas aplicaciones biotecnológicas incluso en condiciones de temperatura extremas.

Palabras clave: Enzima antioxidante; actividad nitrogenasa; daño oxidative; especies reactivas de oxígeno; temperatura.

INTRODUCTION

Ongoing global climate change is estimated to create new biological communities with new species combinations, hence modifying the present complex food web (Mouquet *et al*., 2015). The current scenario of change in global climate has significantly increased the average temperature of the Earth. The present estimates suggest that by the century's end, the planet's average surface temperature may escalate up to 4°C (IPCC, 2013). This gradual upsurge in global temperature may alter the paradigm of optimum temperature needed for the growth of photosynthetic prokaryotes and higher plants, affecting the entire ecosystem (Poli *et al*., 2013). In general, temperature have a major part in environment as it regulates all metabolic and biogeochemical processes of the ecosystem (Raven & Geider, 1988). Numerous physiological and biochemical functions of higher plants and cyanobacteria are temperature-controlled, for instance, growth rate, photosynthesis, respiration, and enzyme activities (Larras *et al*., 2013). Any significant change in temperature above and below the ambient level can influence the morphology and damage the various enzyme-based mechanism which in turn could affect the metabolic processes, an imbalance between photochemistry and metabolism, leading to the overall reduction in growth as well as survivability (Huner *et al*., 1998; Sharkey, 2005).

Temperature stress affects the light reaction, especially "photosystem-II (PS-II)", "thylakoid membrane-embedded protein complex" which catalyses H_2O's oxidation as well as plastoquinone's reduction in plants and cyanobacteria (Renger, 2012) and membrane fluidity, resulting in disturbance in functioning of electron-transport chain, which further initiates *"reactive oxygen species* (ROS)" producing oxidative stress (Halliwell & Gutteridge, 1989). Excess production of ROS causes significant peroxidation of the cell membrane (a site of all metabolic activities), oxidation of protein, causing further reduction in the growth, depending on the extent as well as duration of temperature stress (Imlay & Linn, 1988).

Autotrophic organisms have established various enzymatic as well as non-enzymatic antioxidant defence systems to reduce noxiousness of ROS. However, the extent to which antioxidants can protect against ROS depends on an organism's ability to balance antioxidants' production and scavenge ROS (Chen *et al*., 2018). Enzymatic antioxidant includes SOD which converts singlet oxygen to hydrogen peroxide, AsA peroxidase, and glutathione reductase responsible for removing ROS using AsA-glutathione cycle (Asada, 1999) and CAT. Non-enzymatic antioxidants contain "ascorbic acid", "proline", "α-tocopherol", "glutathione" as well as "β-carotene".

Cyanobacteria are photosynthetic prokaryotes that occupy an extensive range of habitats in both terrestrial and aquatic ecosystems. They were considered as the essential biomass producers on earth (Abed *et al*., 2009), responsible for carrying out around 20-30% of global primary photosynthetic production and fixing atmospheric nitrogen, hence regulating the planet's biogeochemical cycle (Veaudor *et al*., 2020). Therefore, these microorganisms play an imperative role in different agricultural sectors, protecting the environment and water ecology (Singh *et al*., 2016). They are an essential source of primary and secondary metabolites, including certain toxins, biopesticides, pharmaceutical compounds, and growth factors (Abed *et al*., 2009). Cyanobacteria display great potential for biofuel production (Kumar *et al*., 2019) and bio-remediators (accumulation or degradation). They decompose organic waste, detoxify pesticides, heavy metals, and xenobiotics, degrade crude oil (El-Rahman & El-Bestawy, 2002), suppress pathogenic microorganism growth in water and soil, and protect the plants from disease (bio-control agent) (Singh, 2016).

While colonising a wide range of ecosystems, cyanobacteria pass across various environmental stresses, which are likely to influence the growth rate and biochemical composition of these life forms, particularly the temperature-mediated stress factor, which is often associated with the regulation of growth, physiology, and metabolic processes. Although cyanobacteria can adapt to extreme temperature changes, variation in their processes (physiological and biochemical) will modify the food chain and subsequent variation in species composition.

Although the change in cyanobacteria's physiology to temperature stress has been investigated for many cyanobacteria (Jankowiak *et al*., 2019), limited work is available on the comparative account of freshwater and marine water nitrogen-fixing cyanobacteria at different growth temperatures concerning physiological and biochemical activities. Hence, there is a need to inspect the effect of temperatures on physiological as well as biochemical activities of two *Nostoc* species with contrasting habitat types. Further, in this work, we demonstrated a change in the physiological as well as biochemical behaviour of *Nostoc spongiiforme* and *Nostoc calcicola* in response to a broad spectrum of growth temperatures.

MATERIAL AND METHODS

Organisms and growth conditions

Nostoc spongiiforme, a cyanobacterium observed in freshwater, obtained from Dr. Depak Vyas, "Dr. Hari Singh Grover University, Sagar, Madhya Pradesh, India", as well as *Nostoc calcicola,* a marine cyanobacterium, acquired from Bharathidasan University (NFMC), Tiruchirappalli, Tamil Nadu, India. Axenic cultures of *N. spongiiforme* as well as *N. calcicola* have been grown at 25, 30, 35, 40 and 45°C for 13 and 25 days based on their log phase, in nitrogen free BG-11, and ASN III medium respectively (pH 7.6) under fluorescent light of 80μMol photon $m^{-2}s^{-1}$, 16:8 h photo duration in temperature regulated shaker following Stanier *et al*. (1979). Exponentially grown cells of both species were used for the measurement of growth, photosynthetic pigment, lipid peroxidation, peroxidation of protein peroxidation, formation of hydroxyl radical and hydrogen peroxide, antioxidant activities, as well as expression and nitrogenase activity. All experiments had been repeated in triplicate.

Growth estimation

Growth of both *Nostoc* spp., assessed by taking absorbance at 680nm utilizing spectrophotometry (UV-2450, Shimadzu model) as per Wang *et al*. (2012). Approx. 15 min "*Nostoc* culture" (20 ml) has been homogenised in a glass homogeniser to produce a uniform suspension, as well as calculating absorbance at 680nm.

Analysis of photosynthetic pigments

50 mg of tissue has been ground in "80% vol/vol methanol (Merck, HPLC grade)", attaining a final 2 ml volume, kept at 4°C for overnight incubation. The incubated pigment samples had been centrifuged at 4°C for 10 min at 6,700*g*. Supernatants had been collected as well as filtered utilising 0.2 μl Ultipor®N66 ®Nylon membrane filter. The filtered sample of 10 μl had been injected as well as analysed on a high-performance liquid chromatography (HPLC) system (Water, USA). "β-carotene" had been employed as an external standard for relative quantification of photosynthetic pigments (Bhandari & Sharma, 2007).

Analysis of phycobilisome pigments

Phycocyanin (PC) was extracted in 50 m*M* phosphate buffer (pH 6.7) with repeated freezing as well as thawing and centrifuged at 6700 *g* for 15 min. Further, absorbance was measured at 615 as well as 652 nm, employing a UV-Visible spectrophotometer (UV2450, Shimadzu model, Japan). The PC's concentration has been computed employing the following formula according to Sharma *et al*. (2014):

$$PC\ \mu g/ml = \frac{A615 - 0.474\ (A652)}{5.34}$$

Assessment of photosynthetic oxygen evolution

Cyanobacteria cells grown under different temperatures were used for photosynthetic oxygen evolution as well as consumption studies employing a Clark-type oxygen electrode (Oxygraph, Hansatech Instrument Ltd., Norflok, UK). During calculation, homogenised culture as well as 0.1M $NaHCO_3$ into the reaction cuvette, with 2 min of sufficient stirring for oxygen evolution consumption. Photosynthetic rate had been measured by exposing a white light with 1200μMol $m^{-2}s^{-1}$ PAR on the cuvette's surface. The photosynthetic oxygen evolution was expressed in μMoles of O_2 evolved/mg of Chl *a*.

Estimation of total peroxide and hydroxyl radical

The total peroxide content had been evaluated following Sagisaka (1976). Total peroxide production was followed by taking absorbance at 480 nm, and 10 m*M* stock solution of H_2O_2, was used to estimate the total peroxide concentration through a calibration curve and represented as μMol/chl *a* content.

OH content, measured following Liu *et al*. (2009). In this experiment, OH reduced deoxyribose, which forms "thiobarbituric acid reactive substances (TBA-RS)", detected at 532nm (spectrophotometrically). An upsurge in absorbance indicated an increase in hydroxyl radical level. Absorbance units (Absorbance x 1000)/ chl *a* content of sample was used to indicate the concentration of hydroxyl radical.

Measurement of lipid peroxidation (TBARS)

MDA, content had been computed employing “Thiobarbituric acid malonaldehyde (TBA-MDA)” normally used as an indicator for peroxidation of lipid, determined following Bhandari & Sharma (2007). TBA-MDA was obtained at 532nm by employing UV-Visual spectrophotometry. The non-specific turbidity had been amended by deducting absorbance at 600 nm as well as MDA concentration had been computed by extinction coefficient (155 $mM^{-1}cm^{-1}$) as well as MDA content had been expressed as nmol/ chl *a* content.

Measurement of carbonyl content

Protein oxidation was measured by Vega-López *et al*. (2013), with slight modifications. Tissue ground in 30% TCA and spun for 5 min at 1,000 *g*, and supernatant collected. “2,4dinitrophenylhydrazine (DNPH)” (10 mMol) in HCl (2N) to precipitate, followed by vortex for thirty seconds as well as dark-adapted for 1hr, vortexing after every 10 min. Three washing performed to eliminate excess DNPH employing ethanol:ethyl acetate (1:1 V/V), then centrifuged between each wash. Urea of 6 M in 20 mM K_3PO_4 at pH 2.5 used to dissolve the precipitate, and further incubated for 1hr at 37°C, vortex as well as absorbance was recorded at 370nm. The protein carbonyl concentration determined with molar extinction coefficient of 22 $mMol^{-1}cm^{-1}$ as well as reported in nmol carbonyl/mg of proteins.

Estimation of proline and ascorbate content

Proline content in cyanobacterial cells has been valued according the protocol of Bates *et al*. (1973). Three percent sulfosalisilic acid was added to the extracted proline to get it to react with the acid ninhydrin (pH=1.0), resulting in red colour intensity, measured at 520 nm employing “UV-Visible spectrophotometer (2450UV, Shimadzu, Japan)”. Concentration of proline had been quantified employing L-Proline and expressed as μMol/ chl *a* content.

AsA was determined by following Kampfenkel *et al*. (1995). It was extracted with extraction buffer containing HCl (0.1M)+EDTA (0.1 mM). Further, reaction mixture contained, extracted AsA, 0.4M phosphate buffer (pH 7.4), color reagent ([I] H_3PO_4 (15.3%), TCA (4.6%), $FeCl_3$(0.6%) as well as [II] 2,2 dipyridyl (4%) in ethanol, in 2.75:1 ratio) and N-ethylmaleimide (0.5%). Reaction mixture had been incubated at 42°C for 45 min, read at 520nm. AsA (Hi-media), employed standard for quantification as well as expressed as μMol/ chl *a* content.

Estimation of antioxidant enzyme activities

We tested SOD activity (EC 1.15.1.1) by Dhindsa *et al*. (1981). Tissue had been ground up in phosphate buffer (0.5M, pH 7.5) for SOD activity test, centrifuged at 6,700g at 4°C to 10mins, and the supernatant had been employed. Activity of the SOD has been determined through its capacity to stop the photochemical degradation of "NBT (nitro blue tetrazolium)". After being shaken as well as incubated for 15 min under 15 W white light at room temperature, the reaction mixture, which included reaction buffer, 200 mM methionine, 1M Na_2CO_3, 2.25 mM NBT, 3 Mm EDTA, 60 μM riboflavin, and enzyme extract, became light blue. When the reaction mixture and enzyme extract were left in dark, they were regarded as blank A since they did not turn colour. The sample was exposed to light along with a standard that included the reaction mixture except for enzyme extract. Additionally, test tubes had been covered with black cloth and the white fluorescent light was turned off to stop the reaction. Following incubation, a UV-visible spectrophotometer was employed to measure samples' and standards' absorbance at 560 nm in comparison to blank A. The percentage change in colour reduction between the standard and sample was computed. 1unit of SOD activity, represented as Unit/mg of protein/h, was defined as a 50% color reduction. Using the Bradford technique, the protein concentration of the enzyme extract had been computed at 595 nm.

Activity of CAT (EC 1.11.1.6) has been calculated using Aebi's (1984) methodology. In lysis buffer (0.1M K_3PO_4 buffer pH 7.8, PVP (1%), as well as 1 mM EDTA), enzyme had been extracted, and it was centrifuged at 4000 g for 30 min at 4°C. 50mM Na_3PO_4 buffer, pH 7, H_2O_2, along with the enzyme extract were all present in the reaction mixture. At 240 nm, absorbance had been computed employing a "UV-visible spectrophotometer". The unit of enzyme activity was measured in milligrams of protein perminute.

Nitrogenase activity

The activity of Nitrogenase (E.C. 1.18.6.1) had been performed by *"acetylene reduction assay (ARA)"* following Stewart *et al*. (1967) method in both "*Nostoc"* species grown at different growth temperatures of 25, 30, 35, 40 and 45°C in nitrogen free sterile BG-11 and ASN III culture medium. Five milliliters of freshly developed *Nostoc* spp. cultures were placed in a 13 ml glass vial. The cultures had been bubbled with argon gas for approximately 2 min as well as vials were sealed with rubber septa as well as fastened with an aluminum cap employing a seal crimper. Utilising a disposable syringe (26-gauge needle), 10% of the gas phase had been extracted from the vial as well as same amount of acetylene was injected into it.

After that, the vials had been incubated for two hours at 30°C. To stop the enzyme activity, 0.2 ml of 20% trichloroacetic acid was poured into each vial after the incubation time. To determine the concentration of ethylene, 25 μl of gas-phase was extracted from vial as well as introduced into gas chromatography apparatus. Nanomoles of ethylene (C_2H_2)-formed $h^{-1}mg^{-1}$ protein were used to express the findings.

Statistical analysis

Each variable (growth, pigment content, MDA, protein oxidation, total peroxide, $OH^{\cdot}$, SOD, CAT, and nitrogenase activity) was plotted against temperature. All the experiments were conducted in 3 replicates (n=3) and data were mean±SD. Single-factor ANOVA had been carried out employing "XLSTAT Microsoft Excel (version 2020.1.3.65245)" (graphing workspace and data analysis) and graphs were prepared using Origin 8 software, respectively. The statistically significant change has been reported when the P-value≤0.01.

RESULTS

Measurement of growth

Growth of *N. spongiiforme* and *N. calcicola* was measured after 13 and 25 days of the temperature treatment respectively. Both the *Nostoc* species showed better growth (based on OD_{680}) between 25 to 30°C with the mean maximum growth recorded at 30°C. As the temperature raised from 35 to 45°C, there was a significant decrease in the growth of *N. spongiiforme* (17-99%). *N. calcicola* represented a relatively lesser decline in growth (12-84%) as compared to their control (30°C) (Table 1).

Determination of photosynthetic pigments

A significant decline in photosynthetic pigment contents in both "*Nostoc* species" was mainly because of cell death at higher temperature (Table 1). At the extreme temperature (45°C) a reduced chl*a* (96%), Car (81%), and PC (99%) were recorded in *N. spongiiforme* than in *N. calcicola* (74, 76 and 73%), respectively compared to their control.

Table 1. Effect of temperature on the growth, photosynthetic pigments-chlorophyll a (Chla), carotenoids (Car) as well as phycocyanin (PC), and photosynthetic oxygen evolution of *N. spongiiforme* and *N. calcicola* after 13 and 25 days of treatment, respectively. Values are means ± SD, n=3. All treatments were significantly different ($P < 0.01$) from control (Dunnett's two-sided multiple range test); different letters indicate significant differences from each other.

Tabla 1. Efecto de la temperatura sobre el crecimiento, los pigmentos fotosintéticos: clorofila a (Chla), carotenoides (Car), así como la ficocianina (PC) y la evolución fotosintética de oxígeno de *N. spongiiforme* y *N. calcicola* tras 13 y 25 días de tratamiento, respectivamente. Los valores son media ± DE, n=3. Todos los tratamientos presentaron diferencias significativas ($p < 0{,}01$) respecto al control (prueba de rango múltiple bilateral de Dunnett); las letras diferentes presentaron diferencias significativas entre sí.

Nostoc spp.	Temperature (°C)	Growth (680 nm)	Chla, µg/mL cell number	Car, µg/mL	PC, µg/mL	Photosynthetic oxygen evolution, µMol O_2 evolved / (mg Chl *a*)
				Cell number	Cell number	
N. spongiiforme	25	1.27±0.06^f	2.3±0.02^e	1.09±0.01^e	0.42±0.11^d	54.56±9.12^d
	30	1.27±0.01^f	3.1±0.01^f	1.18±0.00^f	0.92±0.07^e	54.27±3.63^d
	35	1.05±0.02^e	1.2±0.01^d	0.75±0.01^c	0.52±0.03^c	41.38±2.23^c
	40	0.08±0.01^b	0.9±0.01^c	0.34±0.01^b	0.31±0.00^{bc}	2.58±0.31^b
	45	0.01±0.01^a	0.1±0.01^a	0.23±0.01^a	0.01±0.00^a	1.05±0.21^a
N. calcicola	25	1.21±0.03^e	6.10±0.01^e	2±0.07^e	1.23±0.03^d	60.71±2.66^e
	30	1.28±0.02^f	7.21±0.01^f	2.14±0.01^f	1.68±0.14^e	54.35±2.51^f
	35	1.13±0.02^d	2.9±0.01^c	1.75±0.30^c	0.99±0.03^c	44.92±5.59^c
	40	0.70±0.02^c	2.7±0.01^b	0.63±0.00^b	0.76±0.04^b	18.59±1.06^b
	45	0.2±0.02^a	1.9±0.01^a	0.52±0.01^a	0.45±0.01^a	7.85±1.01^a

Photosynthetic oxygen evolution measurement

Photosynthetic oxygen evolution was altered with increased temperature stress in both *Nostoc* spp. Oxygen evolution in *N. spongiiforme* decreased gradually from 24-98% between 35-45°C. While in *N. calcicola* a decline of 17-86% at same temperatures had been observed as compared to their control (Table 1).

Determination of ROS generation and oxidative damage

The amount of ROS (total peroxide and OH·) generation and oxidative damage, measured in form of lipid peroxidation as well as carbonyl content, showed a significant increase at extreme temperature (40-45°C), in both the *Nostoc* species (Fig. 1a). MDA content and total peroxide production in *N. spongiiforme* increased progressively from 111-363% and 81-583% respectively. While *N. calcicola* displayed an increase by 105-195% and 76-327% than their control at the same temperatures. Nevertheless, MDA and total peroxide content were 2, 5-fold as well as 2,7-fold higher in *N. spongiiforme* than noted in *N. calcicole* at higher temperatures.

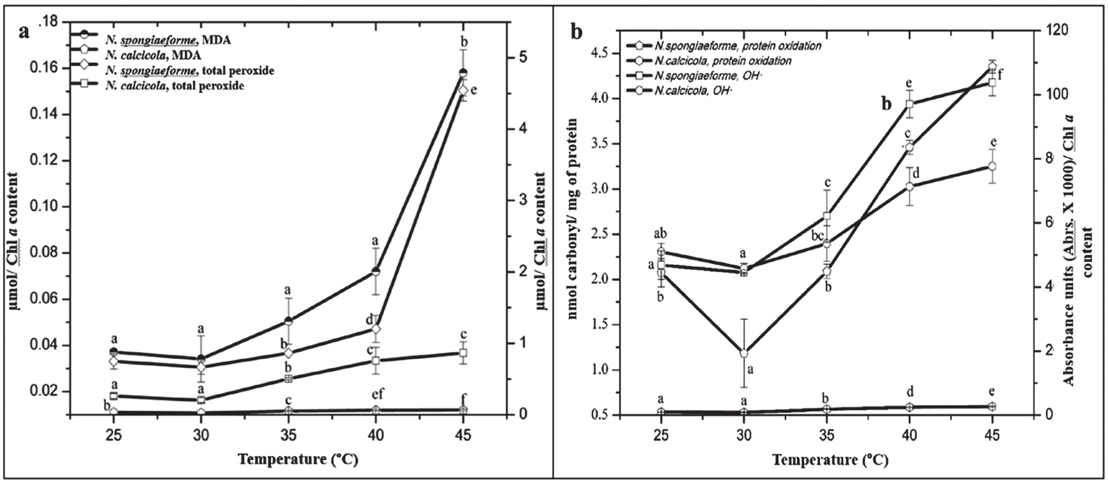

Fig. 1. Temperature effects on MDA, total peroxide (a), protein oxidation, and hydroxyl radicals (b) of *N. calcicola* and *N. spongiiforme*. Vertical bars represent the standard deviation (± SD, n=3). Bars with the same letter indicate an insignificant difference at $p \leq 0.01$.

Fig. 1. Efectos de la temperatura sobre el MDA, el peróxido total (a), la oxidación de proteínas y los radicales hidroxilo (b) de *N. calcicola* y *N. spongiiforme*. Las barras verticales representan la desviación estándar (± DE, n=3). Las barras con la misma letra indican una diferencia insignificante con $p \leq 0,01$.

In the present work, the carbonyl content and hydroxyl radical showed a significant enhancement at 40-45°C in both the *Nostoc* species (Fig.1b). However, 4-fold higher carbonyl content was observed in *N. spongiiforme* than in *N. calcicola* at 40 and 45°C. Values in Fig. 1a, 1b were normalized based on per cell number for ROS generation and oxidative damage.

Estimation of proline and ascorbate content

A notable increase in proline content has additionally been detected in both the *Nostoc* species, because of an increase in temperature stress (Fig. 2a). *N. calcicola* displayed an 8-fold increase with the increasing temperature stress (45°C) compared to their controls. However, in *N. spongiiforme*, proline content increased 2-fold at 45°C in comparison with their control. In contrast to *N. spongiiforme*, proline content in *N. calcicola* is 2-fold higher at 45°C (Fig. 2a). A significant surge in AsA content has additionally been recorded in both *Nostoc* spp., because of temperature stress; though, *N. spongiiforme* displayed 1.5-fold higher AsA content than *N. calcicola* at 45°C (Fig. 2a).

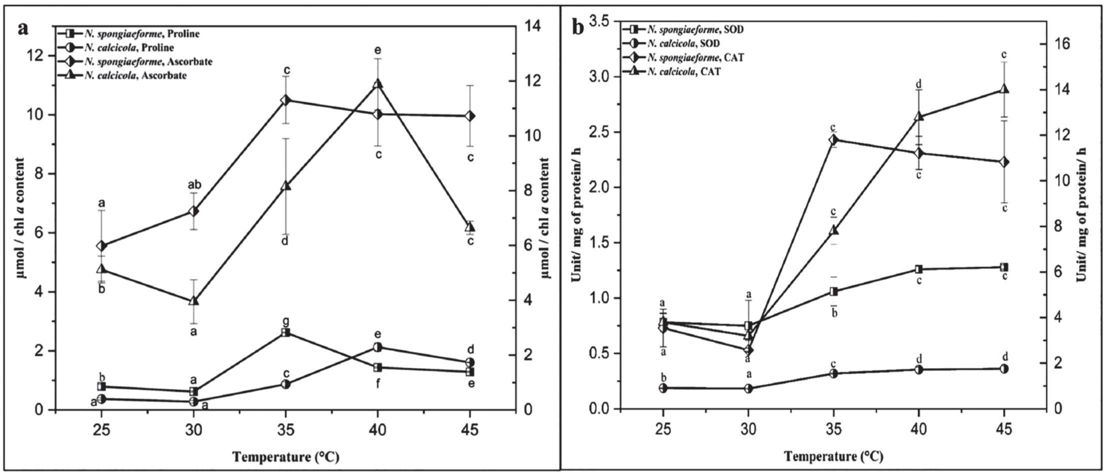

Fig. 2. Effect of temperature on the enzymatic (b) and non-enzymatic (a) antioxidants of *N. spongieforme* and *N. calcicola*. Standard deviation is represented by vertical bars (± SD, n = 3). Bars with the same letter indicate an insignificant difference at $p \leq 0.01$.

Fig. 2. Efecto de la temperatura sobre los antioxidantes no enzimáticos y enzimáticos de N. spongieforme y N. calcicola. La desviación estándar se representa mediante barras verticales (± DE, n=3). Las barras con la misma letra indican una diferencia insignificante con $p \leq 0{,}01$.

Superoixde Dismutase(SOD) and Catalase(CAT) activities

SOD activity of *N. calcicola* has been higher than *N. spongiiforme* in response to extreme higher temperature. It increased linearly because of temperature stress in *N. spongiiforme* by 106% at 45°C, on contrary *N. calcicola,* showed 98% higher SOD activity at same temperature compared to their mean optimum temperature (Fig. 2b).

Data related to CAT activity in both *Nostoc* spp., under temperature stress, have been demonstrated in (Fig. 2b). The activity of the CAT in both the *Nostoc* spp., was 0.53±0.02 and 3.2±0.2 Unit/mg protein/min at optimum mean temperature. The extremely high temperature stimulated the CAT activity in both the "*Nostoc* spp."; however, "*N. calcicola*" displayed 6-fold higher CAT activity in comparison to *N. spongiiforme.*

Determination of nitrogenase activity

Figure 3 displays information about the nitrogenase activity in both *Nostoc* species under temperature stress. In contrast to the ideal temperature of 30°C, both *Nostoc* species in this investigation displayed a decline in nitrogenase activity at supra-optimal temperatures. When grown at supra-optimal temperatures of 35, 40, and 45°C, *N. spongiiforme* was shown to exhibit a larger decline in nitrogenase activity than *N. calcicola*.

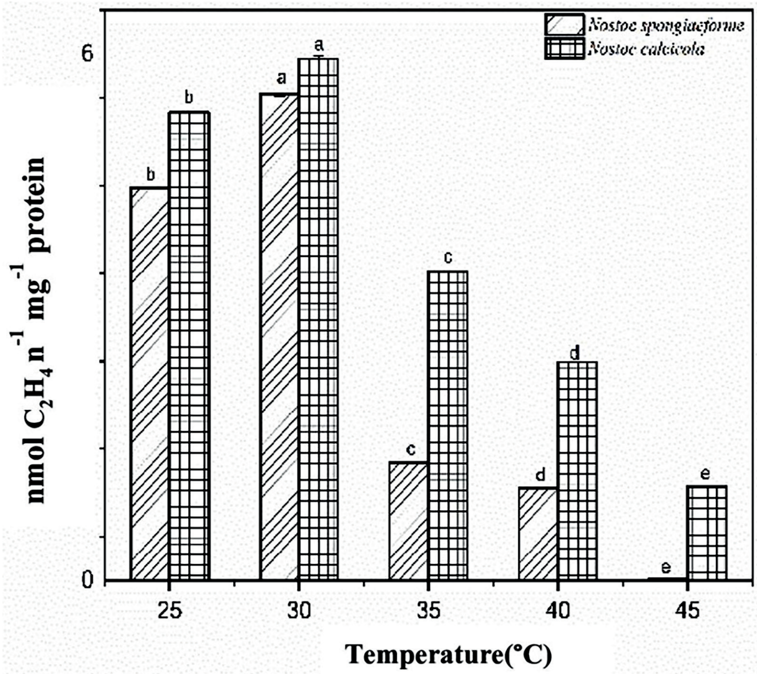

Fig. 3. Nitrogenase activity *of N. calcicola* and *N. spongiaeforme* as a function of growth temperature. ± SD, n=3 are vertical bars that indicate standard deviation. Bars with the same letter indicate an insignificant difference at $p \leq 0.01$.

Fig. 3. Actividad nitrogenasa de *N. calcicola* y *N. spongiaeforme* en función de la temperatura de crecimiento. ± DE, n=3 son barras verticales que indican la desviación estándar. Las barras con la misma letra indican una diferencia insignificante con $p \leq 0{,}01$.

When compared to the ideal temperature of 30°C, nitrogenase activity in *N. spongiiforme* and *N. calcicola* dropped by 76, 81, and 97%, respectively, and 41, 58, and 82%, respectively, as a result of the temperatures of 35, 40, and 45°C.

DISCUSSION

Based on the study, temperature-induced disturbance in cellular homeostasis leads to an increase in the production of ROS to a level much higher than their scavenging potential, resulting in oxidative damage as exhibited by lipid peroxidation and carbonyl content. All these amendments finally affect the growth as manifested in Table 1. The decreasing trend in growth with increasing temperature have been because of filament's shrinkage as well as breakage, leading to a negative alteration in structural integrity of membrane (data not shown). The supra-optimal temperature might lower enzyme's cell metabolic activities, specially "ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco)" (Wei *et al*., 2015), leading to an imbalance between ATP production as well as energy demand (Leggat *et al*., 2004), therefore declined growth in both *"Nostoc* spp*"* (Table.1).

Supra-optimal temperature causes suppression in the photosynthetic pigments and photosynthetic rate in both *"Nostoc* species" and hence, causes a decline in the growth performance (Table 1). A parallel research observed by (Renaud *et al*., 2002), who reported a significant decline in growth rates of four cyanobacteria species when grown at extreme temperatures which could be because of decline in the activity of photosynthetic enzymes and disruptions in the cell membrane metabolisms (Davison *et al*., 1991). Several other studies (Wu *et al*., 2013) have also reported the impacts of extreme temperature on growth of different cyanobacteria species. Nevertheless, both the *Nostoc* species in the present study registered relatively higher growth rates at intermediate temperature levels 25–30°C), which is in par with the results of (Renaud *et al*., 2002; Wu *et al*., 2013). Therefore, it is obvious from the current study that the growth of *Nostoc* spp., irrespective of their habitat types, can derive an advantage on growth when exposed to the optimal temperature, ranging between 25–30°C. However, the extent of variations in growth between the two species differed significantly, especially the *N. calcicola* showed greater tolerance to the changing temperature than *N. spongiiforme*. Similar contrasting growth performance of two species of cyanobacteria grown at the same temperature were reported by (Renaud *et al*., 2002), thus the growth of cyanobacteria species to the varying temperature can be species-specific (Thompson *et al*., 1992). Photosynthetic pigments have been core part of energy indicator of nearly all photosynthetic organisms and consequently any significant change in their level may amend overall metabolic activities and growth status.

A significantly more reduction in the O_2 evolution in *N. spongiiforme* than in *N. calcicola* might be interrelated with the decrease in the pigment content (Table 1) as detected in current research. A similar decrease in the pigment content under high temperature stress was also reported by (Kłodawska *et al*., 2019; Zhu *et al*., 2020) which supported the above study. Such a decrease in the pigment content may be attributed to the inhibition of photosynthetic enzymes activities under temperature stress or augmented degradation of pigments because increased ROS production at various sites of photosynthetic electron transport chain throughout temperature stress (Zhang & Lui, 2016). Carotenoid being one of accessory pigments play key role in protecting chl *a* and PC along with thylakoid membrane from oxidative damage. Hence, decrease in its content might have severe consequences on other two photosynthetic pigments; Chl *a* and PC as well as on photosynthetic membrane which might be responsible for the reduction in the photosynthetic efficiency of the *N. spongiiforme* more than in *N. calcicola.*

By measuring the degree of lipid peroxidation (Fig. 1a) as well as protein oxidation (Fig. 1b), we were able to demonstrate that the observed increase in ROS concentration in preaent research at supra-optimal temperature indicated oxidative damage in both *Nostoc* species.

Since ROS are primarily produced in the photosynthetic system as a result of over-energization as well as reduction of light reaction as well as pigments have been extremely sensitive to such oxidative damage, the depigmentation in both *"Nostoc* species*"* at supra-optimal temperatures may also be caused by oxidative damage to chlorophyll molecules in LHC (Zheng *et al*., 2020). Furthermore, our findings on the activity of antioxidant enzymes at supra-optimal temperature (Table 2) further support this conclusion, most likely to shield the cell from ROS.

A remarkable development of defensive mechanisms (enzymatic and non-enzymatic) against temperature induced damage to membrane lipid and protein, because of their activities of antioxidants (Fig. 2) in temperature tolerant *N. calcicola*, which did not suffer much because of stress in comparison to *N. spongiiforme,* leading to much more production of carbonyl content and peroxidation of lipid in *N. spongiiforme* than *N. calcicola.* In current research high accumulation of proline content following temperature treatment could be because of the generation of free radical which might have resulted an upsurge in glutamate kinase activity causing an increase in proline biosynthesis (Shamim *et al*., 2017). *N. calcicola* accumulated 6-fold additional proline in cells under temperature treatment than in control conditions (Fig. 2a). In conclusion, though proline over production under temperature stress conditions is an imperative response of stress, a higher level of proline accumulation as well as a high critical point discloses a higher temperature tolerant in "*N. calcicole".* An increase in the AsA content in *N. calcicola* because of positive correlations with reduced H_2O_2 and MDA content could be because of signalling properties of nitric oxide, involved in antioxidant defence system (Begara-Morales *et al*., 2016).

Subsequently, a higher SOD activity escorted by higher CAT activity in *N. calcicola,* could be suggested that both the enzymatic antioxidants work more efficiently to quench ROS generation in temperature-tolerant *N. calcicola* resulting in better growth as well as lesser oxidative damage in comparison to temperature-sensitive *N. spongiiforme.* Our data are in par with (Reddy *et al*., 2019) in *Anabaena doliolum* as well as (Han *et al*., 2015) in *Microcystis aeruginosa.* Thus, current work approves a correlation between antioxidant defence mechanism as well as tolerance towards oxidative damage encouraged by elevated temperature in the *N. calcicola.*

Nitrogen fixation is a temperature-sensitive process and signifies a significant input of nitrogen to tropical ecosystem (Thangaraj *et al*. 2017). Our data showed that temperature had strong effect on nitrogenase activity of mesophilic cyanobacteria from freshwater (*N. spongiiforme*) as well as marine habitat (*N. calcicola*). For both the *Nostoc* species nitrogenase activity positively responded between 25-30°C, with maximum activity recorded at 30°C. However, further rise in temperature above 30°C negatively affected the activity of nitrogenase enzyme of both the *Nostoc* spp (Fig. 3).

Among 2 studied *Nostoc* spp., *N. calcicola* exhibited better nitrogenase activity than *N. spongiiforme* under temperature stress. *Nostoc* spp. can fix nitrogen at an optimum temperature, related to their better growth and photosynthesis at this temperature as nitrogen fixation is an energy-demanding process and thus highly reliant on the process of photosynthesis for ATP and reducing power, which was maximum at optimum temperature. The photosynthesis parameters of both *Nostoc* species, however, were adversely affected at supra-optimal temperatures. This resulted in a decrease in nitrogen fixation because a greater percentage of fixed nitrogen might be released as well as not incorporated into cells, either because of insufficient carbon fixation or because of increased membrane permeability under temperature stress. Additionally, tightly entangled trichomes (heterocysts) have a multilayered coating that creates an anaerobic environment to shield the nitrogenase enzyme from harsh environments. The heterocysts may have been harmed by temperature stress, which resulted in oxygen diffusion and nitrogenase enzyme deactivation. Stal (2017) reported a linear rise in nitrogen fixation up to a temperature of 36°C in short-term incubations, which is in contrast to our results of an optimal temperature of 30°C. Similarly, Thangaraj *et al*. (2017) observed maximum nitrogenase activity at 37°C and least at 4°C in two mesophilic *Nostoc* strains. Stal, (2017) showed that a thermophilic cyanobacterium *Fischerella sp.* exhibited an increase in nitrogenase activity till 50°C when grown between 12°C to 60°C, and above 50°C, nitrogenase activity collapsed. Reddy *et al*. (2019) also reported a significant reduction in nitrogenase activity of *Anabaena doliolum* under elevated temperature (35-40°C).

CONCLUSION

In the current research, it has been evident that the temperature will have a significant effect on the photosynthetic as well as biochemical properties of *Nostoc* strains, irrespective of their habitat types. Further, it is also demonstrated that the extent of the impact of temperature can be species-specific, as both species produced contrasting responses when exposed to a similar temperature. However, we recommend the need for including more species of cyanobacteria to have better insights. This study is of utmost importance, considering the progress in global climate change and increasing temperature which could have a significant impact on such life forms and overall species diversity. Further research will help in screening of both *Nostoc* strains to find out about the genes responsible for helping these cyanobacteria to survive at high temperature (especially *N. calcicola*) so they can be cultured in bulk and used for developing various usable products for humans in the coming years.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest between themselves or against third parties

AUTHOR CONTRIBUTIONS

The author contributed to research design as well as data analyses, had been revised, edited as well as approved the final manuscript.

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