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Research Article

Effect of Various Nitrogen Sources on Heterocyst Frequency in *Nostoc punctiforme* (Kütz.) Har.

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Abstract

The study examined the effect of various nitrogen sources on heterocyst frequency in *Nostoc punctiforme* using BG-11 medium. Various concentrations of sodium nitrate, calcium nitrate, potassium nitrate, ammonium nitrate, and urea were tested. The results indicated that nitrogen-deficient conditions promoted higher heterocyst frequency (10.16%), compared to nitrogen-supplemented environments, while ammonium nitrate and urea exhibited inhibitory effects (0%). Other sources, such as sodium, calcium, and potassium nitrates, have varying effects, with sodium nitrate (1.5 g/L in BG-11 medium) yielding a frequency of 5.74%. Morphological analyses revealed adaptations in filament structure related to nitrogen availability. The nitrogen supplementation resulted in longer, less twisted filaments and a reduced presence of heterocysts. Findings emphasize the regulatory role of nitrogen sources in heterocyst formation, providing insights into the ecological adaptability of cyanobacteria to nitrogen availability, with implications for agricultural practices, biofertilization and environmental management.

Keywords: *Ammonium Nitrate; Cyanobacteria; Heterocyst Frequency; Nitrogen Sources; Nostoc punctiforme*

Introduction

Understanding nitrogen fixation in cyanobacteria, particularly in species such as *Anabaena* and *Nostoc*, necessitates exploring how various nitrogen sources influence heterocyst (the specialized cells enabling these organisms to fix atmospheric nitrogen) development. Wherein, investigations reveal that fluctuating nitrogen availability is vital for their survival in nitrogen-limited environments. The impact of different nitrogen sources, including ammonium, nitrate, and atmospheric nitrogen (N_2), on heterocyst frequency and formation in cyanobacteria varies on species and nitrogen concentration [1]. Research indicates that ammonium can inhibit heterocyst development, while nitrate sources promote it under certain conditions. For instance, *Anabaena variabilis* showed high growth rates with ammonium but limited heterocyst formation, suggesting a complex relationship between nitrogen source and cellular growth [2]. Furthermore, the lowest heterocyst numbers were observed with ammonium-nitrogen and the highest with atmospheric nitrogen [3]. They also found that heterocyst production depends on the absence of combined nitrogen and the presence of phosphate, which also highlights the intricate relationship between nitrogen sources and heterocyst development, which can vary across species and environmental conditions. Nitrogen deprivation generally triggers heterocyst production for nitrogen fixation, and the assimilation of different nitrogen sources is regulated at the gene expression level, with a preference for more reduced nitrogen forms, where transcription factors like NtcA, PacR, and HetR are crucial for regulating nitrogen assimilation and heterocyst differentiation in response to carbon and nitrogen availability [1]. Additionally, the presence of nitrogenous compounds can stimulate the growth of heterotrophic bacteria, potentially harmful when using cyanobacteria as biofertilizers. Also, water quality can affect cyanobacterial growth and heterocyst frequency, with some strains performing better in

distilled water compared to tap water [4]. In *Nodularia spumigena,* high ammonium levels were found to decrease nitrogen fixation activity and *nifH* gene expression, whereas nitrate had no significant effect. Nevertheless, heterocyst frequency and *hetR* gene expression were maintained in *N. spumigena* even when nitrogen fixation ceased [5].

The morphology of *Nostoc* filaments is significantly influenced by various nitrogen sources, leading to distinct structural adaptations that include alterations in cell differentiation, filament structure, and the presence of specialized cells such as heterocysts. Specifically high concentrations of $NaNO_3$ and NH_4Cl inhibit heterocyst formation, resulting in long filaments primarily composed mainly of vegetative cells, while lower concentrations allow for some heterocyst development, indicating a direct relationship between nitrogen source concentration and filament morphology. In nitrogen-rich environments, vegetative cells accumulate large cyanophycin granules, essential for nitrogen storage and filament integrity [6], whereas under nitrogen starvation, there is a marked increase in heterocyst differentiation crucial for nitrogen fixation, leading to changes in filament width and cell wall structure [7]. In contrast, while nitrogen availability promotes certain filamentous structures, nitrogen starvation can lead to extensive differentiation and morphological changes that enhance survival in nutrient-poor conditions. This duality highlights the adaptability of *Nostoc* to varying environmental nitrogen levels [7]. In addition, *Cylindrospermopsis raciborskii*, demonstrates growth rates that are highest with ammonium and lowest without fixed nitrogen, with cylindrospermopsin concentrations showing the opposite trend. Different nitrogen sources also affect cyanobacterial morphology, with ammonium causing loss of heterocysts and tapering of end cells [8]. Furthermore, in *Anabaena naviculoides*, potassium nitrate and sodium nitrate enhance growth and akinete development, shortening the period of akinete differentiation compared to control cultures [9]. Collectively, these findings underscore the complex relationship between nitrogen sources, heterocyst formation, and nitrogen fixation in cyanobacteria, emphasizing the need for species-specific considerations in research and applications with implications for cyanobacterial taxonomy and potential applications in agriculture and biotechnology.

Materials and Methods

Cyanobacterial culture conditions

The *Nostoc punctiforme* culture, acquired from the UTEX culture of Algae, located in Austin, Texas, USA, was grown in 250 mL conical flasks containing 100 mL of BG-11(+) medium with nitrogen and BG-11(-) medium without nitrogen, both at pH 7.5 [10]. The cultures were maintained at 28 ± 2°C under a 16:8h of light: dark cycle with cool white fluorescent lights under 400 µmol photons $m^{-2}s^{-1}$ of irradiance in culture rooms. Additionally, one mL of exponentially growing cell cultures (with an OD_{730} = 0.2) was taken to conduct all experiments, which were performed in triplicate.

Heterocyst frequency modulation with different nitrogen sources

BG-11 medium was experimented with different nitrogen sources, including $NaNO_3$, $Ca(NO_3)_2$, KNO_3, NH_4NO_3 and urea, to modulate heterocyst frequency; while heterocyst formation occurred with $NaNO_3$, concentrations above and below 1.5 g/L were tested, and for the other nitrogen sources, concentrations of 0.150 g/L, 0.200 g/L, 0.253 g/L, 0.300 g/L were used, with heterocyst frequency observed under a compound microscope on the 10th day of treatment.

Heterocyst frequency measurement

The heterocyst formation in *N. punctiforme* was examined using an Olympus CH20i compound microscope, and the heterocyst frequency was measured by counting at least 500 cells, expressed as a percentage of the total cell count [11].

$$\text{Heterocyst frequency} = \frac{\text{No. of heterocysts cell}}{\text{Total no. of cells}} \times 100$$

Morphological analysis of *Nostoc punctiforme* filaments

The morphological changes in *N. punctiforme* cells were examined on the 10th day of the experiment to check for heterocyst formation and any contamination under the Olympus CH20i compound microscope for both control and treated cells.

Statistical analysis

Experiments were conducted in biological triplicate to ensure reproducibility and the results were presented as the mean value ± standard deviation (SD) of the mean using MS-Excel version 16.8.

Results

Effect of different nitrogen sources on heterocyst frequency

The frequency of heterocysts was influenced by various nitrogen sources in the BG-11 medium, with a nitrogen-deficient control medium exhibiting the highest heterocyst frequency compared to the nitrogen-supplemented medium (Table 1). However, the addition of ammonium nitrate and urea inhibited heterocyst formation, with ammonium nitrate being the most effective since it resulted in zero heterocyst formation and stable cultures, while sodium nitrate, calcium nitrate, and potassium nitrate did not inhibit heterocyst formation. Although their higher concentrations than those shown in table 1 ultimately led to cell death.

Morphological behaviour of *Nostoc punctiforme* filaments

Under nitrogen-deficient conditions, *Nostoc punctiforme* showed a higher frequency (Figure 1) of heterocyst development, indicating a morphological adaptation to nitrogen scarcity. The use of ammonium nitrate and urea as a nitrogen source inhibited heterocyst formation, which resulted in longer filaments with many vegetative cells that were narrower, lacked heterocysts, and exhibited reduced filament twisting and aggregation. Also, large cyanophycin granules were present, indicating a shift in metabolic focus towards storage rather than nitrogen fixation. Conversely, while calcium, sodium, and potassium at a concentration of 0.253 g/L (Table 1) promoted heterocyst formation with elongated cells with increased width, twisted filaments and tapering ends (Figure 2).

Nitrogen source in BG-11 Medium	Effective optimum concentration	Day	Heterocyst Frequency (%)
With no nitrogen (Control)	-	10	10.16
Sodium nitrate	1.5 g/L	10	5.74
Calcium nitrate	0.253 g/L	10	6.42
Potassium nitrate	0.253 g/L	10	4.36
Ammonium nitrate	0.253 g/L	10	0
Urea	0.253 g/L	10	0

***Table 1**: Response of different nitrogen sources on heterocyst frequency.*

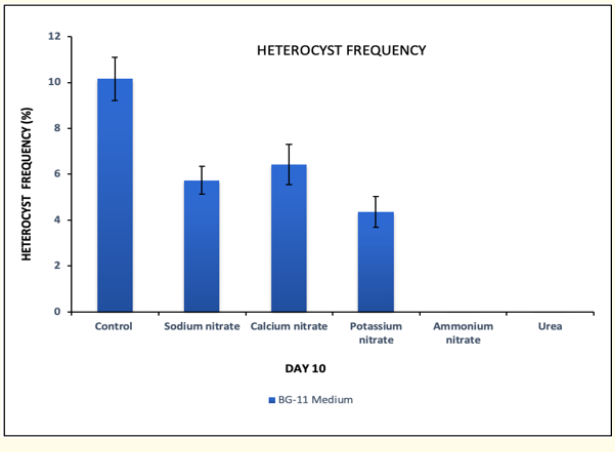

***Figure 1:** The graph illustrates the percentage of heterocyst frequency in BG-11 medium, which is supplemented with different nitrogen sources, while the control represents the condition without any nitrogen source.*

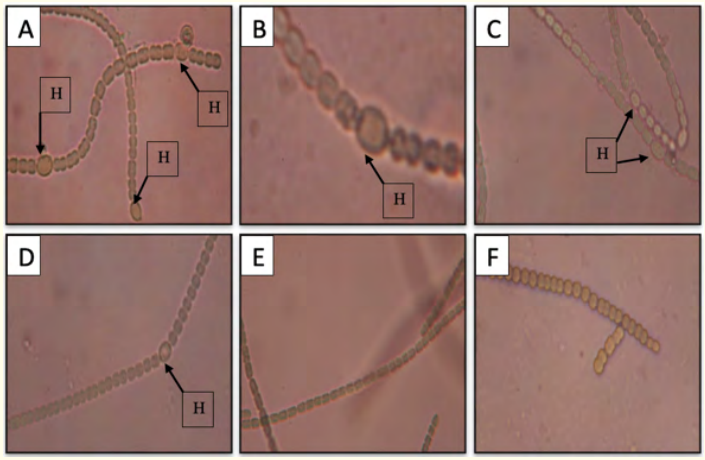

Figure 2: *Morphological behaviour of Nostoc punctiforme filaments under different nitrogen sources in BG-11 medium - specifically no nitrogen (A) sodium nitrate (B), calcium nitrate (C) potassium nitrate (D), ammonium nitrate (E), and urea (F). Here 'H' in the images denotes heterocysts.*

Discussion

The formation of heterocysts even under sodium nitrate conditions is attributed to the complex regulatory mechanisms that oversee their development. Sodium nitrate, typically a non-reducing nitrogen source for heterocyst formation in cyanobacteria, paradoxically promotes heterocyst differentiation under certain conditions. The presence of sodium (1.5 g/L), potassium (0.253 g/L), and calcium nitrates (0.253 g/L) promotes heterocyst formation in cyanobacteria, whereas urea and ammonium nitrate at 0.253 g/L inhibit this process. In a study, sodium nitrate does not inhibit heterocyst production in *Anabaena* sp. L-31, unlike potassium nitrate. The presence of sodium nitrate allows for ammonia release, which, when exceeding a threshold, depletes intracellular ammonia levels, triggering heterocyst induction [12]. The *patS* gene, which inhibits heterocyst differentiation, is expressed in response to nitrogen availability; in environments rich in ammonium or urea, *patS* expression is upregulated, preventing heterocyst formation [13]. Nitrates can serve as a nitrogen source that stimulates heterocyst formation when combined with specific ions like sodium, potassium, and calcium. These ions may enhance the metabolic processes necessary for heterocyst differentiation. In the presence of nitrate, gene regulation involved in heterocyst differentiation is altered, allowing heterocyst formation even when nitrogen is available [14], which confirms the results presented in this study. Conversely, more reduced nitrogen forms, such as ammonium and urea, suppress heterocyst formation because these organisms prioritize assimilating readily available nitrogen sources over developing specialized nitrogen-fixing cells. This represents a trade-off between structural adaptation and functional performance in cyanobacteria. Moreover, while the type of nitrogen source influenced heterocyst formation, its concentration was crucial as high nitrate levels reduced the development. In contrast, lower concentrations permitted some formation with morphological modifications in filaments, as shown in the results. These findings underscore the adaptability of cyanobacteria to varying nitrogen conditions, which is vital for their ecological success and potential applications in sustainable agriculture and biofertilization. Although excessive nitrogen sources can cause eutrophication, negatively impacting aquatic ecosystems. These findings are significant for the application of nitrogen-fixing cyanobacteria in agriculture and environmental management.

Conclusion

This study provided information on the change in heterocyst frequency in the presence of different nitrogen sources and identified the optimum concentration that was tolerable to the cells without inhibiting the growth. Also, higher nitrogen concentrations were found to be toxic. Additionally, further research could explore how ammonium nitrate inhibits heterocyst formation, whereas other nitrogen sources in BG-11 medium still support some heterocyst presence.

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