The Pharma Innovation Journal 2023; 12(9): 1568-1573

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(9): 1568-1573

www.thepharmajournal.com
Received: 10-07-2023
Accepted: 14-08-2023

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Unveiling the mechanisms of salinity tolerance in cyanobacteria through copper pre-treatment

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Abstract
In the current work, the cyanobacterium *A. doliolum* was pre-exposed to copper chloride (5 µM) for a range of time duration—24 hours, 48 hours, and 72 hours—and then treated with doses of 150 and 200 mM NaCl to better understand how pre-exposure modifies the physiologic activities of the *A. doliolum*. The findings show that the test cyanobacterium's cellular components did not significantly change after being pre-exposed to copper chloride for 24 and 48 hours. However, the pre-exposure produced an enhanced profile of proline and antioxidant enzymes. The findings demonstrated the detrimental effects of salinity and copper on the development of the cyanobacterium *A. doliolum* as well as the significance of pre-exposure in the control of the cellular antioxidant machinery.

Keywords: Cyanobacterium, pre-treatment, salinity stress, copper stress, antioxidants

1. Introduction
Cyanobacteria are Gram-negative autotrophic bacteria with an array of different metabolic capabilities and adaptation processes. They possess the remarkable capacity to fix nitrogen, which takes place in specialized cells called heterocytes either through temporal spatiation or microaerobic environments. In addition to chromatic adaptability, cyanobacteria may coexist harmoniously with a range of eukaryotic hosts, including plants, fungi, and protists. Along with accessory pigments including phycobilins, carotenoids, and xanthophylls, the main photosynthetic pigment in these bacteria is chlorophyll a. Notably, the origin of the photosynthetic organelles known as plastids, which have had a significant influence on the development of life on Earth, is thought to have resulted from an endosymbiotic interaction between a cyanobacterium and a eukaryote (Tashyreva and Elster, 2016) [28].

Salinity has an impact on agricultural yield. Higher inorganic ion concentrations can impair the cellular development and metabolism of cyanobacteria (Huang *et al*., 2006) [14], yet cyanobacteria are significantly more salt stress tolerant than higher plants (Wei *et al*., 2006) [30]. When the concentration of Na^+ ions in the cytoplasm of cyanobacterial cells increases, cyanobacteria generally follow restricted sodium absorption and active sodium outflow via Na^+/H^+ antiport as a regulatory mechanism (Molitor *et al*., 1986) [21]. The cyanobacterial cell also employs the following mechanisms when exposed to Na^+ toxicity: 1) gathering organic molecules to maintain a constant osmoticum; 2) activating the antioxidant defense system to detoxify reactive oxygen species; and 3) inducing salt-inducible proteins. As a result of these modifications, the flow of inorganic ions into the periplasm is probably reduced and a strong diffusion barrier is created (Allakhverdiev *et al*., 2002) [2].

Since cyanobacteria engage in oxygenic photosynthesis, they usually need a much greater concentration of metals than non-photosynthetic organisms. Cyanobacterial plastocyanin and cytochrome oxidase both contain copper (Cu) (Tchounwou *et al*., 2012) [29]. By using various kinds of strategies, such as membrane modifications, biotransformation, transfer pump activation or inactivation, and sequestration (Hantke, 2005) [12], cyanobacteria protect metallic homeostasis. The production of reactive oxygen species (R.O.S.) at greater Cu concentrations, however, can have an impact on normal physiology (Xu *et al*., 2010) [30]. Additionally, too much copper can interfere with a cell's ability to photosynthesize and maintain its redox balance, which can damage the ultrastructure of the cell and ultimately cause it to die (Yadav *et al*., 2022) [6].

However, cyanobacteria may encounter a multitude of challenges in the natural environment or may be subject to one or more stressors at any given moment and we are unsure of the combined impact of factors like salinity and heavy metals on cyanobacteria.

It has been proven that different stresses can change an organism's susceptibility to a specific stress (Bhargawa *et al*., 2008) [5]. *Cylindrospermum* sp., a cyanobacterium, has been shown to have increased resistance to UV-B as a result of prior UV-B exposure (Chris *et al*., 2006) [7]. Copper pre-treatment improved the UV-B tolerance in the cyanobacterium *Anabena doliolum* (Bhargawa *et al*., 2008) [5]. Cyanobacterium was employed by Kumar and Gaur (2014) [15] to remove copper from copper-contaminated water bodies. There have been no findings on how copper pre-treatment affects the cyanobacteria's ability to tolerate salt, despite the fact that salinity poses a major hazard to both plants and cyanobacteria. Therefore, it is crucial to comprehend how the stress response is modulated in cyanobacterium that has been subjected to salt and copper stress.

2. Material and Methods

2.1 Experimental organism and growth conditions: For the current research, the diazotrophic cyanobacterium *Anabaena doliolum* was employed, which is regularly grown at the Centre for Conservation and Utilization of BGA, ICAR-Indian Agricultural Research Institute, New Delhi, India. This bacterium was routinely cultivated in unialgal form in BG-11 medium without additional nitrogen. The growth medium's pH was set to 7.5, and it received 72 µmol m^{-2} s^{-1} of light per second at a temperature of 28±2 °C during a 16/8 h light/dark cycle.

2.2 To understand the modulation of salinity tolerance in cyanobacterium through copper pre-treatment: Pre-exposure to copper chloride (5 M) was done on the cyanobacterium for several periods of time: 24 hours, 48 hours, and 72 hours. The pre-exposed cyanobacterium is then given a NaCl treatment (150 and 200 mM). The following treatment combinations have been designed.

Treatment Details

T_1 = Control (0 mM NaCl)
T_2 = 150 mM NaCl
T_3 = 200 mM NaCl
24-hour pre-treatment with 5 µM $CuCl_2$
T_4= 150 mM NaCl
T_5 = 200 mM NaCl
48-hour pre-treatment with 5 µM $CuCl_2$
T_6= 150 mM NaCl
T_7 = 200 mM NaCl
72-hour pre-treatment with 5 µM $CuCl_2$
T_8 = 150 mM NaCl
T_9 = 200 mM NaCl

2.3 Dry weight: After proper stirring, a known quantity of the cyanobacterial suspension (10 ml) was filtered using Whatman No. 42 filter paper. The cultures were then oven dried at 60 °C and chilled until constant weight was attained in a desiccator. The difference in weights was noted as dry weight (Sorokin, 1973) [24].

2.4 Protein Content Estimation: The technique of Lowry *et al*. (1951) [16] was used to determine the protein content of the cyanobacterium.

2.5 Pigment profile

a) Chlorophyll a: The cold extraction technique (McKinney, 1941) [17] was employed to ascertain the total chlorophyll concentration.

b) Carotenoids: The MacKinney (1941) [17] approach was used to determine the cyanobacteria's carotenoid content.

c) Phycocyanin: Using Bennett and Bogorad's (1973) [4] approach, the cyanobacteria's phytocyanin content was calculated.

2.6 Total Carbohydrate: The total carbohydrate content of the cyanobacterial samples was calculated using Spiro's (1966) [25] method.

2.7 Estimation of average filament length: A little quantity of the cyanobacterial suspension was pipetted out and placed on a clean grease-free slide. Under a low-power microscope, the filaments were focussed. The number of cells (vegetative cells and heterocysts) in each filament in a field was counted. The average length of a filament in a sample was calculated by counting the number of cells in each filament and dividing by the total number of cells (Mishra, 2003) [20].

2.8 Estimation of heterocyst frequency: The number of heterocysts per hundred vegetative cells is known as heterocyst frequency, and it was determined as follows by Mishra (2003) [20]:

$$\text{Heterocyst frequency} = \frac{\text{Total number of heterocyst}}{\text{Total number of vegetative cells}} \times 100$$

2.9 Assay of enzymatic and non-enzymatic antioxidant enzymes

a) Superoxide dismutase: It was determined by measuring the reduction in optical density of formazone caused by superoxide radical and nitro-blue tetrazolium dye (Dhindsa *et al*., 1981) [8].

b) Ascorbate peroxidase: It was measured using an approach of Nakano and Asada, 1981 [23].

c) Catalase: Catalase enzyme activity was assayed by monitoring the decrease of H_2O_2 Aebi (1984) [1].

d) Proline content: The proline content of the cyanobacterial samples was determined using the Bates *et al*. (1973) [3] methodology.

3. Results

Effect of copper pre-treatment on the growth, cellular constituents, and antioxidant enzyme activity of the cyanobacterium *A. doliolum*

The relevance of the pre-exposure of the organism to copper chloride and its role in improving the salinity tolerance of the cyanobacterium *A. doliolum* was investigated in the current study. This was accomplished by first pre-exposing the test cyanobacterium to copper chloride (5 M) at periods of 24, 48, and 72 hours. Following that, the pre-exposed cyanobacterium was subjected to NaCl concentrations of 150 and 200 mM.

The effect of pre-exposure to copper on the development of the cyanobacterium *A. doliolum* is shown in Table 1 as an increase in dry weight. Pre-exposure to copper for 24 hours resulted in a modest increase in dry weight when compared to cells treated directly to 150 mM NaCl. A comparable rise was found after 48 hours of copper treatments. However, pre-exposure to copper for 72 hours had no significant effect on growth. The protein content of the cyanobacterium *A.*

doliolum that had been pre-treated with copper increased (Table 2). In contrast, 72-hour pre-treatment resulted in a poor reaction when compared to immediate NaCl exposure.

The photosynthetic pigment Chlorophyll a was also observed to respond favorably to 24 and 48 hours of pre-exposure, however, the response was not statistically significant (Table 3). However, the accessory pigment phycocyanin increased slightly in response to pre-exposure to copper (Table 4). However, the carotenoid concentration responded positively to pre-exposure (Table 5). Pre-exposure had no effect on heterocyst frequency or average filament length, regardless of duration (Table 6). The sugar content of the cyanobacterium *A. doliolum* in response to pre-exposure is shown in Table 7. The sugar concentration surged considerably after 24 hours and 48 hours of pre-exposure. The sugar concentration increased after 72 hours of pre-exposure.

The impact of pre-exposure to copper on nitrogen assimilation enzymes was investigated (Table 8). Although direct exposure increased nitrate reductase activity, pre-exposure had no effect on nitrate reductase activity. However, the pre-treatment had no effect on glutamine synthase or nitrogenase activity. The antioxidant profile of the test cyanobacterium is significantly influenced by pre-exposure (Table 9). Copper pre-treatment for 24 and 48 hours greatly increased the activity of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, and catalase. The buildup of proline was also found to be considerably impacted by pre-exposure. Pre-exposure resulted in an increase in proline content when compared to immediate exposure.

As a result, the findings of this study clearly demonstrated that pre-exposure to copper had a role in providing salt tolerance to the cyanobacterium *A. doliolum*. Cyanobacterial cells treated to short-duration pre-exposure of 24 hours and 48 hours were shown to be beneficial, however exposure to 72 hours had no effect on the cyanobacterium's salt tolerance. Although growth and other cellular components did not considerably increase, antioxidant enzymes responded strongly to pre-exposure and after salt therapy.

Table 1: Effect of pre-exposure to copper on the dry weight accumulation ($mgml^{-1}$) of the cyanobacterium *Anabaena doliolum* further exposed to salinity

Treatments	4th day	8th day	12th day
T1	1.36±0.0153	1.86±0.02	1.98±0.0152
T2	1.16±0.0152	1.36±0.0153	1.39±0.0153
T3	0.868±0.00208	0.614±0.0115	0.426±0.03
T4	1.18±0.01	1.48±0.767	1.56±0.0152
T5	0.876±0.0208	0.624±0.00252	0.434±0.01
T6	1.26±0.153	1.56±0.0148	1.64±0.020
T7	0.872±0.03	0.628±0.00208	0.438±0.035
T8	1.12±0.0135	1.41±0.0115	1.46±0.015
T9	0.856±0.0153	0.616±0.00153	0.618±0.005

Table 2: Effect of pre-exposure to copper on the Protein (µg mg^{-1} dry weight) content of the cyanobacterium *Anabaena doliolum* further exposed to salinity

Treatments	4th day	8th day	12th day
T1	46.1±0.30	64.4±0.37	66.4±0.17
T2	32.4±0.20	40.1±0.20	44.4±0.15
T3	12.6±0.15	18.1±0.26	18.6±0.15
T4	34.6±0.24	46.8±0.66	48.1±0.31
T5	12.8±0.43	18.6±0.15	19.1±0.15
T6	37.1±0.26	50.1±0.21	51.4±0.23
T7	13.2±0.26	18.8±0.28	19.4±0.26
T8	30.6±0.27	32.4±0.24	34.1±0.16
T9	11.8±0.15	12.6±0.17	13.1±0.27

Table 3: Effect of pre-exposure to copper on the Chlorophyll a content (µg mg^{-1} dry weight) of the cyanobacterium *Anabaena doliolum* further exposed to salinity

Treatments	4th day	8th day	12th day
T1	3.06±0.02	3.66±0.021	4.04±0.025
T2	2.46±0.04	2.64±0.013	2.84±0.012
T3	0.806±0.002	0.821±0.04	0.828±0.335
T4	2.58±0.026	2.76±0.036	2.84±1.489
T5	0.808±0.028	0.826±0.04	0.834±0.052
T6	2.64±0.025	2.88±0.031	2.86±0.153
T7	0.810±0.031	0.816±0.034	0.818±0.041
T8	2.42±0.033	2.46±0.035	2.48±0.351
T9	0.801±0.035	0.804±0.041	0.807±0.02

Table 4: Effect of pre-exposure to copper on Phycocyanin content (µg mg^{-1} dry weight) of the cyanobacterium *Anabaena doliolum* further exposed to salinity

Treatments	4th day	8th day	12th day
T1	0.032±0.02	0.042±0.05	0.046±0.03
T2	0.026±0.02	0.032±0.05	0.036±0.01
T3	0.012±0.01	0.016±0.01	0.018±0.015
T4	0.030±0.01	0.038±0.02	0.044±0.0153
T5	0.014±0.02	0.018±0.005	0.019±0.02
T6	0.037±0.05	0.046±0.02	0.048±0.03
T7	0.012±0.01	0.014±0.01	0.015±0.015
T8	0.032±0.028	0.036±0.01	0.037±0.02
T9	0.009±0.001	0.012±0.01	0.012±0.015

Table 5: Effect of pre-exposure to copper on Caroteniod (µg mg^{-1} dry weight) the Cyanobacterium *Anabaena doliolum* further exposed to salinity.

Treatments	4th day	8th day	12th day
T1	2.41±0.01	2.68±0.04	2.82±0.02
T2	3.16±0.04	3.89±0.02	4.28±0.02
T3	2.86±0.0155	3.16±0.015	3.24±0.03
T4	3.86±0.0153	4.26±0.01	4.68±0.025
T5	3.14±0.025	3.72±0.015	3.90±0.0208
T6	3.46±0.025	3.92±0.017	4.14±0.02
T7	3.18±0.153	3.28±0.01	3.62±0.03
T8	3.21±0.252	3.20±0.0208	3.25±0.036
T9	2.90±0.03	2.96±0.03	3.02±0.04

Table 6: Effect of pre-exposure to copper on Heterocyst Frequency and Average Filament Length of the cyanobacterium *Anabaena doliolum* further exposed to salinity

Treatments	HF	AFL
T1	15.8±0.1	54.2±0.208
T2	10.1±0.2	42.1±0.2
T3	3.6±0.153	16.4±0.1
T4	10.4±0.1	41.6±0.208
T5	3.4±0.153	16.2±0.265
T6	9.1±0.208	41.8±0.208
T7	3.8±0.265	16.1±0.306
T8	9.6±0.1	40.8±0.208
T9	3.1±0.1	15.8±0.2

Table 7: Effect of pre-exposure to copper on the sugar (µg mg^{-1} dry weight) content of the cyanobacterium *Anabaena doliolum* further exposed to salinity

Treatments	4th day	8th day	12th day
T1	32.1±0.231	48.4±0.361	50.6±0.153
T2	40.4±0.265	60.1±0.306	62.4±0.153
T3	38.6±1.353	52.4±0.3	54.5±0.217
T4	44.5±0.231	64.1±0.208	65.1±0.243
T5	40.2±0.263	54.8±0.252	56.1±0.306
T6	48.6±0.208	68.1±0.306	70.1±0.608
T7	44.1±0.306	56.4±0.209	58.4±0.252
T8	42.5±0.153	57.2±0.361	59.4±0.153
T9	40.1±0.309	51.7±0.208	52.6±0.153

Table 8: Effect of pre-exposure to copper on the N assimilation enzymes of the cyanobacterium *Anabaena doliolum* further exposed to salinity

Treatments	Nitrate reductase (µmol mg protein $^{-1}$)	Glutamine Synthetase (nmol mg protein $^{-1}$)	Nitrogenase (µmol C_2H_4 mg chl^{-1} h^{-1})
T1	6.21±0.047	172.4±0.557	32.4±0.351
T2	14.71±0.041	148.4±0.36	20.6±0.1
T3	10.8±0.1	120.1±0.71	11.2±0.208
T4	14.2±0.208	146.4±1.27	20.8±0.153
T5	9.6±0.1	121.4±0436	11.8±0.2
T6	14.8±0.2	144.5±0.208	21.2±0.321
T7	10.2±0.252	118.2±0.529	12.4±0.153
T8	15.1±0.351	148.8±0.265	22.8±0.265
T9	10.4±0.1	124.2±0.153	10.8±0.154

Table 9: Effect of pre-exposure to copper on the antioxidant profile of the cyanobacterium *Anabaena doliolum* further exposed to salinity.

Treatments	Superoxide dismutase (U mg protein^{-1})	Ascorbate peroxidase (U mg protein^{-1})	Catalase (U mg protein^{-1})	Proline (µ mol mg protein^{-1})
T1	1.36±0.15	1.94±0.01	2.78±0.0153	32.6±0.252
T2	1.96±0.1	2.46±0.0252	4.92±0.0321	46.8±0.208
T3	2.01±0.20	2.96±0.0153	4.96±0.01	40.4±0.473
T4	2.46±0.2	2.68±0.03	5.16±0.0252	54.6±0.3
T5	2.12±0.265	3.04±0.03	5.04±0.0173	42.8±0.436
T6	2.86±0.152	3.12±0.0321	5.28±0.0208	60.4±0.643
T7	2.16±0.153	3.06±0.0252	5.08±0.0404	44.2±0.351
T8	2.04±0.252	2.44±0.0208	5.20±0.0252	50.1±0.416
T9	2.06±0.153	2.86±0.0252	5.01±0.0361	41.4±0.513

4. Discussion

To determine if pre-exposure to one stress had any influence on the other, the cells were pre-treated with copper for several time periods. The pre-treatment data indicated a distinct trend in terms of growth factors as well as antioxidants. A 24-hour pre-treatment with copper chloride followed by salinity led in a small improvement in dry weight, protein, chlorophyll, phycocyanin, phycoerythrin, and allophycocyanin. When the cyanobacterium was pre-treated to copper chloride for 48 hours before being exposed to copper chloride, a more or less identical reaction was seen.

Surprisingly, the sugar and carotenoid levels spiked dramatically in response to pre-exposure for 24 and 48 hours. However, the pre-exposure had little effect on the nitrogen absorption enzymes. It has been established that organisms' susceptibility to specific stress may be affected by additional stresses (Bhargava *et al*., 2008) [5]. Murali and Teramura (1987) [22] discovered that drought and mineral scarcity can alter the UV-B impact on plants. Pre-exposure to UV-B has been shown to improve UV-B resistance in the cyanobacterium *Cylindrospermum* sp. (Chris *et al*., 2006) [7]. Bhargava *et al*. (2008) [5] discovered that copper pre-treatment improved UV-B tolerance in the cyanobacterium *Anabena doliolum*.

The pre-exposure showed a favorable effect on the antioxidant enzymes. Salt tolerance has been linked to increased antioxidant enzyme activity (Sreenivasulu *et al*., 2000) [26]. Signaling molecules such as H_2O_2 activate genes that govern oxidative stress, such as APX and SOD, and cause cross-adaptation in maize seedlings (Gong *et al*., 2001) [11]. Hernandez *et al*. (1995) [13] proposed that salt stress increased m-RNA expression and activity of APX and SOD. Copper pre-treatment for 72 hours had no effect on any of the measures evaluated. Copper pre-exposure appears to have some adaptive importance, most likely via antioxidant enzymes and proline buildup. The copper-induced accumulation of proline, as well as the hyperactivity of antioxidant enzyme activities, may inhibit free radical generation and reduce lipid peroxidation.

Our findings reveal that essential physiological factors linked to antioxidant defense and nitrogen metabolism increased in cells pre-treated with copper for 24 and 48 days. This clearly shows that the cyanobacterium became conditioned after being exposed to low quantities of copper, which is regarded as a key adaptive approach. Pre-exposure does, in fact, result in priming effects. Priming prepares the cyanobacterium to strengthen its defensive capabilities for future threats. Through the stimulus of priming, cellular machinery is communicated physiological, transcriptional, metabolic, and epigenetic changes in organisms, such as altering the composition of membranes, inducing specific transport processes (intracellular homeostasis), and appropriately metabolic changes that are essential for their defense (Mauch-Mani *et al.*, 2017) [19]. Martinez-Medina *et al*., (2016) [18] and Mauch-Mani *et al*., (2017) [19] discovered that these defenses were more resilient and performed better than directly generated defenses while requiring negligible to minimum cost, allowing the cyanobacterium to develop normally. Furthermore, it was established that priming is long-lasting and may be passed down from generation to generation (Mauch-Mani *et al*., 2017) [19]. As a consequence of the findings, it appears that the priming stimuli send signals to the cellular machinery, inducing suitable cellular processes to cope with stress by alerting them.

5. Conclusion

In this study, the cyanobacterium's response to pre-exposure to copper chloride and subsequent treatment with varying concentrations of NaCl was investigated. The findings reveal a nuanced and adaptive strategy employed by the cyanobacterium to cope with mild copper stress. Pre-exposure for 24 and 48 hours resulted in a significant enhancement of antioxidant enzyme activity and an increase in intracellular proline accumulation, indicative of an effective defense mechanism against potential oxidative stress caused by copper. However, this adaptive response seemed to have a temporal limitation, as pre-exposure for 72 hours failed to yield similar benefits. Furthermore, nitrogen assimilation enzymes remained unaffected by copper pre-exposure. These results highlight the cyanobacterium's ability to fine-tune its physiological responses to environmental stressors, showcasing its resilience and adaptability in the face of changing conditions, which may have broader implications for understanding the ecological roles of cyanobacteria in diverse ecosystems.

6. Author Contributions

Conceived and designed the analysis, Collected the data, Performed the analysis, and wrote the paper: Shivaranjan, C.S., and Dr. Gerard Abraham.

7.Acknowledgment

The authors acknowledge the Laboratory facilities supported at the Division of Microbiology, Indian Agricultural Research Institute, New Delhi, to conduct the experiments.

8. Conflict of interest

None

9. References

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