Research Article

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Oxidative stress and response of antioxidant system in *Nostoc muscorum* exposed to different forms of Zinc

Farklı Çinko formlarına maruz bırakılan *Nostoc muscorum*‘da oksidatif stres ve antioksidan sistemin tepkisi

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Abstract

Objective: Present study aims to evaluate oxidative stress-mediated toxicity mechanisms of nano-ZnO (N-ZnO) compared to ZnCl₂ and bulk-ZnO (M-ZnO), using cyanobacteria as a toxicity assessment tool.

Methods: *Nostoc muscorum* was used as a test organism and the experiments were performed in parallels after treated with 2–128 μM of N-ZnO, M-ZnO, and ZnCl₂.

Results: Low concentrations of ZnCl₂ (2–8 μM) and N-ZnO (16–32 μM) led to a significant increase in the carotenoids, which could be ascribed to its role as an antioxidant, whereas higher concentrations were inhibitory to the photosynthetic pigments. Enhanced activities of superoxide dismutase (SOD), peroxidase (POD) and accelerated lipid peroxidation depicted significant accumulation of superoxide and peroxide radicals in *N. muscorum* exposed to N-ZnO and ZnCl₂. The non-enzymatic antioxidants such as proline and total phenols also showed enhanced accumulations up to 16 and 64 μM of ZnCl₂ and N-ZnO treatment, respectively.

Conclusion: Our results for the very first time depicted the significant accumulation of stress biomarkers in *N. muscorum* in response to deleterious effects of oxidative stress caused by N-ZnO, ZnCl₂, and M-ZnO. Present results revealed varying levels of toxicity of various forms of zinc, which decreased in an order of ZnCl₂ > N-ZnO > M-ZnO.

Keywords: Cyanobacteria; Photosynthetic pigments; Oxidative stress; SOD; POD; Proline; Phenolic compounds; Zinc oxide nanoparticles; Zinc chloride.

Özet

Amaç: Bu çalışmada, toksisite değerlendirme aracı olarak siyanobakteri kullanarak ZnCl₂ ve bulk-ZnO (M-ZnO) ile karşılaştırıldığında, nano-ZnO’in (N-ZnO) oksidatif stres aracılı toksisite mekanizmasını değerlendirmediyiz amaçladık.

Yöntemler: Bir test organizması olarak *Nostoc muscorum* kullanılmıştır, deneyler parallel olarak 2–128 μM N-ZnO, M-ZnO ve ZnCl₂ ile muamele edildikten sonra gerçekleştirmiştir.

Bulgular: Düşük konsantrasyonda ZnCl₂ (2–8 μM) ve N-ZnO (16–32 μM) antioksidan rolünü destekleyen bir şekilde, karotenoidlerde belirgin bir artış göstermiştir, bunun yanı sıra daha yüksel konsantrasyonlar fotosentetik pigmentleri inhibe etmiştir. Süperoksid dismutaz (SOD), peroksidaz (POD) aktivitelerindeki artış ve hızlandırılmış lipit peroksidasyonu, N-ZnO ve ZnCl₂’ye maruz
Introduction

Our environment is being routinely exposed to a variety of contaminants including heavy metals due to unrestricted developmental activities, which might pose a serious threat to our ecosystem [1]. Besides these heavy metal contaminants, the rapid expansion of nanotechnology has also resulted in the subsequent significant release of nanoparticles (NPs) into the aquatic environment during the cycle of manufacturing, transportation, consumption, and disposal [2]. Amongst all the known heavy metal and metal nanoparticles, zinc oxide nanoparticles (ZnO NPs) have gained great attention in recent years due to its wide application for both industrial and household applications [3]. They are widely used as UV-absorbers, catalyst and nanosensors due to their unique optical, catalytic, semiconducting, piezoelectric, and magnetic properties [4]. In the presence of excessive amounts of metals, the toxicity symptoms appear as a result of interactions at the cellular/molecular level. The scale of the problem has prompted the search for effective and reliable ways of monitoring the toxic effects of these pollutants. To determine the toxicity of these contaminants various bioassays are used, in which wide range of organisms are being recognized as a bioindicator for predicting the possible mechanism of toxicity and the underlying mechanisms to combat the subsequent oxidative stress [5]. The use of photosynthetic prokaryotes like cyanobacteria as a bioindicator is significant because they form the base of the aquatic food chain and any alterations in their populations can alter the upper trophic level of the ecosystem. These primary producers are widely distributed in various habitats particularly copious in marine ecosystems and foreseen as an ideal model to study the toxic effects of the released NPs [6], as they are effective biological metal sorbents and have the ability to accumulate many metals in a significant amount. They rapidly respond and adapt to stress condition in general and in particular of heavy metal (Ni) stress [7].

Metal nanoparticle-mediated toxicity might be due to one or combination of several effects like coordination effects, genotoxicity, non-homeostasis effects and oxidative stress. Amongst these, the most important mechanism is ROS mediated toxicity once they interact or get internalized into the living cells depending upon their solubility, which is a key issue in the toxicity of metal nanoparticles [8]. Due to the large surface area of NPs and higher electronic density as compared to their bulk counterpart they interact with biomolecules with enhanced reactivity [9]. To combat the effect of oxidative stress triggered by excessive ROS generation, cells possess enzymatic and non-enzymatic antioxidant defence mechanisms. Enzymatic components include peroxidase (POD), catalase (CAT), ascorbate, superoxide dismutase (SOD) and some important non-enzymatic components are total phenol and proline. In response to various stressful conditions such as osmotic stress, high salinity and heavy metal stress intracellular proline showed enhanced accumulation to combat the resulting oxidative stress [10].

From the present study, it is concluded that Nostoc muscorum can tolerate ZnCl₂ up to a concentration of 8 μM, N-ZnO up to 32 μM and M-ZnO up to the highest chosen concentration (128 μM). Thus, this blue-green alga can not only be used as a cost-effective metal/metal nanoparticles toxicity assessment tool but also for bioremediation in the aquatic ecosystem.

Materials and methods

Chemicals and reagents

All the chemicals were purchased from a standard company like SD-fine, Sigma-Aldrich, Merck (Worli, Mumbai), Glaxo, Fischer, Himedia, etc.

Experimental organism and growth conditions

Cyanobacterium, N. muscorum used in the present study as a test organism was obtained through the courtesy of Dr S. M. Prasad, Department of Botany, University of Allahabad. The axenic culture of N. muscorum was maintained in the plant tissue culture room at 27 ± 2°C under the light...
intensity of 2400 Lux and 14:10 h light and dark photo-period. Cultures were grown in 1 L BG11 media (pH 7.0) [11] for regular experiments.

**Stock preparation of ZnCl₂, M-ZnO, and N-ZnO for treatment**

ZnCl₂, M-ZnO and N-ZnO (<100 nm), all were purchased from Sigma-Aldrich. The stock suspensions/solutions (1000 μM) of the tested chemicals were prepared in deionized water (Milli-Q, Millipore). All stock suspensions except ZnCl₂ were sonicated (Ultrasonic Bath sonicator, Model- 3.5 L 100 H, electric supply- 230 V AC 50 Hz, Qualigens) for 30 min, stored in the dark at +4°C and used for testing within 2 months. The M-ZnO and N-ZnO suspension were again sonicated for 15 min immediately prior to each treatment.

**Experimental design**

An exponential phase culture of *N. muscorum* was supplemented with the stock solution of N-ZnO to give the selected concentrations (2, 4, 8, 16, 32, 64 and 128 μM) in the test medium. Simultaneously, *N. muscorum* was also treated with similar concentrations (2–128 μM) of two reference molecules (ZnCl₂ and M-ZnO) to better understand the underlying toxicity mechanisms of N-ZnO into the cells. Thereafter, selected parameters such as growth measurement, pigment estimation, total peroxide, superoxide, lipid peroxidation, SOD, POD, proline, and phenol content were estimated as per the methods described below. All experiments were conducted in triplicate and repeated thrice to confirm the reproducibility of the results.

**Growth measurement**

Growth was measured in terms of chlorophyll estimation on alternate days at regular intervals for 10 days.

**Photosynthetic pigment extraction and estimation**

For extraction of chl-a and carotenoids, an equal volume of *N. muscorum* was centrifuged and the pellet was suspended in 80% acetone (acetone: water, v/v). For chl-a and carotenoids estimation, the absorbance of extracts was read spectrophotometrically (Systronics 2020) at 665 nm and 480 nm, respectively. The content of both the pigment was calculated by the specific coefficients given by Myers and Krats [12]. The quantitative estimation of pigments in terms of g/L was done by the formula given below:

\[
C = D / d \alpha
\]

where, \(\alpha\), absorption coefficient (value of \(\alpha\) for chl-a is 82.04 and for carotenoids is 200); D, optical density; d, inside path length of spectrophotometer in (cm); C, concentration of pigment in gL⁻¹.

**Estimation of protein**

Protein content was measured by the method developed by Lowry et al. [13]. For protein estimation, cyanobacterial cells were digested with 0.5 mL of 1 N NaOH for 10 min in a boiling water bath. Thereafter, 2.5 mL of reagent C was added and incubated for 15 min at room temperature followed by addition of 0.5 mL of Folin-Ciocalteu’s reagent. The absorbance of resulting blue color was taken at 660 nm. The concentration of protein in μg/mL was calculated from the BSA standard curve.

**Superoxide estimation**

Superoxide radical was estimated in ZnCl₂, M-ZnO and N-ZnO treated and untreated cells of *N. muscorum* as per the method of Elstner and Heupel [14]. Cells were homogenized in 2 mL phosphate buffer and centrifuged at 8000×g. Thereafter, 0.9 mL phosphate buffer and 0.1 mL hydroxylamine were added in 1 mL of supernatant. O.D was recorded at 530 nm after 20 min incubation with 1 mL of each NEDD and sulfanilamide. A standard curve of sodium nitrite was used for the calculation of superoxide content.

**Peroxide estimation**

The level of peroxide in the cells was determined by following the method of Sagisaka [15]. For peroxide estimation, treated and untreated cells were extracted in 3.5 mL of 5% trichloroacetic acid (TCA). After that, total peroxide in the supernatant was recorded spectrophotometrically at 480 nm. The amount of total peroxide was calculated by referring to H₂O₂ standard curve.

**Lipid peroxidation**

Lipid peroxidation in the ZnCl₂, M-ZnO, and N-ZnO treated and untreated cells of *N. muscorum* were measured by
estimating the end product malondialdehyde (MDA) as per the method of Heath and Packer [16]. The cellular pellets were homogenized in 50 mM phosphate buffer. The resulting homogenate was centrifuged at 8000xg for 20 min. To 0.5 mL aliquot of the supernatant, 2 mL of 20% trichloroacetic acid (TCA, w/v in TBA solution) containing 0.5% thiobarbituric acid (TBA, w/v in 0.2 N HCl) was added. The mixture was heated at 90°C for 20 min and then quickly cooled in ice bath. After centrifugation at 8000xg for 10 min, the absorbance of the supernatant was read at 532 nm. The value for nonspecific absorption of each sample at 600 nm was also recorded and subtracted from the absorption recorded at 532 nm. The concentration of MDA, an end product of lipid peroxidation was calculated from extinction coefficient 155 m mol⁻¹ cm⁻¹. The result is represented as nmol MDA/g dry weight.

Superoxide dismutase activity (SOD)

SOD activity was measured spectrophotometrically by the method of Giannopolitis and Ries [17]. The unit of superoxide dismutase activity was defined as the amount of enzyme which caused a 50% inhibition of the reaction observed in the absence of enzyme.

Peroxidase activity (POD)

For the estimation of the POD, the method of Gahagen et al. [18] was followed. Treated and untreated cells were homogenized in 2 mL of 100 mM phosphate buffer (pH 7.0) at 5°C. After that, the homogenate was centrifuged at 8000xg for 30 min. One millilitre of enzyme extract was mixed with 1 mL of 25 mM H₂O₂ and 1 mL of 100 mM pyrogallol prepared in deionized water. After mixing, change in optical density was recorded for 2–3 min at 430 nm.

Proline content measurement

Proline was determined by following Bates et al. [19] method. For proline estimation 15 mL treated and untreated culture was centrifuged at 8000xg. Then, the cells were homogenized in 10 mL of 3% sulfosalicylic acid and again centrifuged at 8000xg to remove cell debris. Thereafter, 2 mL of acid ninhydrin and glacial acetic acid was added to 2 mL of obtained supernatant and incubated for 1 h at boiling temperature. The toluene was used for extraction of proline into an organic phase, which was used for quantification at 520 nm. A standard curve of proline was used for the calculation of proline concentration.

Phenol content measurement

Total phenol content was estimated by the method of Singleton and Rossi [20]. For the total phenol extraction, 10 mL of treated and untreated cells were centrifuged and extracted in 2 mL of 80% methanol. After that, 2 mL sodium bicarbonate, 0.3 mL double-distilled water (DDW) and 0.2 mL Folin reagent was added in 0.5 mL of supernatant and incubated in a water bath until the blue color developed. The absorbance of the colored compound was recorded at 750 nm. A Gallic acid standard curve was used for estimation of total phenol.

Statistical analysis

All experiments were performed using exponentially growing cultures and repeated three times to ascertain the reproducibility of the results. Values are represented as mean ± SEM (n = 3). Tests of significance were performed by one-way analysis of variance (ANOVA), followed by Dunnett’s test with control by using Graph Pad Prism 5 software.

Results and discussion

Metal and metallic nanoparticles toxicity to cyanobacteria are one of the most threatening environmental problems. Zinc is essential to trace elements for the living organisms, but at higher concentrations, it can cause severe damages even the cell death.

Figure 1A–C demonstrate the effects of different concentrations (2–128 μM) of ZnCl₂, N-ZnO, and M-ZnO, respectively on the growth pattern of N. muscorum. When the cells of N. muscorum was treated with 2–8 μM of ZnCl₂, the lag phase occurred for 2 days followed by a log phase which continued up to 10 days. Thereafter, the dose-dependent decline in growth can be observed after the 8th and 2nd day, once treated with 16–32 μM and 64–128 μM of ZnCl₂, respectively (Figure 1A). The most toxic form of zinc, i.e. Zn²⁺ become toxic only at higher concentrations [21]. Whereas, after treated with a similar dose of N-ZnO (2–128 μM), the lag phase occurred for 2 days followed by a log phase which continued up to 10 days. Thereafter, the dose-dependent decline in growth can be observed after the 8th and 2nd day, once treated with 16–32 μM and 64–128 μM of N-ZnO, respectively (Figure 1B). The most toxic form of zinc, i.e. Zn²⁺ become toxic only at higher concentrations [21]. Whereas, after treated with a similar dose of N-ZnO (2–128 μM), log phase continued up to 10 days for 2–32 μM of N-ZnO. The remarkable decline in growth was observed only at higher concentration (64 and 128 μM) of N-ZnO after the 6th day of treatment (Figure 1B). On the other hand, no significant alteration in the growth pattern of N. muscorum was observed after treated with 2–128 μM of M-ZnO up to 10 days as compared to control (Figure 1C). Our results are in consonance with [22], who also showed
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the dose-dependent (0.05, 0.1, 0.2, 0.42 and 1 mg/L) and time-dependent (24, 48 and 72 h) growth inhibition in two algae sp. *Scenedesmus dimorphus* and *Chlorella Vulgaris* treated with ZnO NPs. The decline in the growth of cyanobacteria under various abiotic stresses including heavy metal stress has been reported earlier [23]. Results presented in Tables 1–3 clearly illustrate the photosynthetic pigments and total protein of *N. muscorum*

**Table 1:** Effect of ZnCl₂ on photosynthetic pigments and total protein of *N. muscorum* measured after 72 h of treatment.

| ZnCl₂ (μM) | Chl-a (μg mL⁻¹)   | Carotenoids (μg mL⁻¹) | Total protein (μg mL⁻¹) |
|------------|--------------------|-----------------------|-------------------------|
| 0          | 3.7 ± 0.26         | 1.33 ± 0.07           | 94.4 ± 0.31             |
| 2          | 4.8 ± 0.28 (+29)ᵃ  | 2.2 ± 0.17 (+65)ᵇ     | 97.1 ± 1.0 (+3)         |
| 4          | 5.4 ± 0.28 (+45)ᵇ  | 2.9 ± 0.11 (+118)ᶜ    | 107.2 ± 2.9 (+13)       |
| 8          | 3.6 ± 0.29 (2)     | 1.96 ± 0.18 (+47)ᶜ    | 122.3 ± 2.0 (+29)ᵃ      |
| 16         | 3.4 ± 0.26 (9)     | 1.2 ± 0.08 (10)       | 130.8 ± 0.5 (+39)ᵇ      |
| 32         | 3.1 ± 0.20 (16)    | 0.9 ± 0.14 (32)       | 106.6 ± 1.5 (+12)       |
| 64         | 2.8 ± 0.23 (24)    | 0.7 ± 0.12 (47)ᶜ      | 89.1 ± 2.5 (6)          |
| 128        | 1.9 ± 0.17 (49)ᶜ   | 0.3 ± 0.05 (77)ᶜ      | 60.7 ± 1.9 (36)ᵇ        |

Values in parenthesis are [%] decrease or increase (+). The results represented are the mean ± SEM of three independent experiments (n = 3) performed in triplicate (*p < 0.05, ‡p < 0.01, §p < 0.001 represent significant difference compared with control).
Table 2: Effect of N-ZnO on photosynthetic pigments and total protein of *N. muscorum* measured after 72 h of treatment.

| N-ZnO (μM) | Chl-a (μg mL⁻¹) | Carotenoids (μg mL⁻¹) | Total protein (μg mL⁻¹) |
|------------|-----------------|-----------------------|------------------------|
| 0          | 3.7 ± 0.26      | 1.33 ± 0.07           | 94.4 ± 0.31            |
| 2          | 3.9 ± 0.28 (+5) | 1.37 ± 0.05 (-3)      | 95.2 ± 0.2 (+0.8)      |
| 4          | 4.1 ± 0.27 (+11)| 1.4 ± 0.05 (+5)       | 96.2 ± 0.4 (+2)        |
| 8          | 4.4 ± 0.26 (+19)| 1.49 ± 0.05 (-12)     | 97.4 ± 0.7 (+3)        |
| 16         | 4.6 ± 0.23 (+26)| 1.62 ± 0.03 (-21)     | 99.3 ± 2.9 (+16)       |
| 32         | 5.4 ± 0.30 (+45) | 1.71 ± 0.01 (-28)     | 133.2 ± 2.1 (+41)      |
| 64         | 5.1 ± 0.28 (+37)| 1.4 ± 0.01 (-5)       | 107.4 ± 2.3 (+14)      |
| 128        | 3.5 ± 0.17 (5)  | 0.9 ± 0.11 (32)       | 76.6 ± 2.7 (19)        |

Values in parenthesis are [%] decrease or increase (+). The results represented are the mean ± SEM of three independent experiments (n = 3) performed in triplicate (*p* < 0.05, *p* < 0.01, *p* < 0.001 represent significant difference compared with control).

Table 3: Effect of M-ZnO on photosynthetic pigments and Total Protein of *N. muscorum* measured after 72 h of treatment.

| M-ZnO (μM) | Chl-a (μg mL⁻¹) | Carotenoids (μg mL⁻¹) | Total protein (μg mL⁻¹) |
|------------|-----------------|-----------------------|------------------------|
| 0          | 3.7 ± 0.26      | 1.33 ± 0.07           | 94.4 ± 0.31            |
| 2          | 3.8 ± 0.20 (+3) | 1.37 ± 0.06 (-3)      | 95.0 ± 0.14 (+0.6)     |
| 4          | 3.89 ± 0.17 (+5)| 1.4 ± 0.06 (+4)       | 95.4 ± 0.21 (+1)       |
| 8          | 4.1 ± 0.17 (+11)| 1.43 ± 0.06 (+5)      | 96.2 ± 0.36 (+2)       |
| 16         | 4.29 ± 0.18 (+16)| 1.46 ± 0.069 (+10)   | 96.9 ± 0.2 (+3)        |
| 32         | 4.36 ± 0.18 (+18)| 1.49 ± 0.06 (+12)    | 97.4 ± 0.5 (+3)        |
| 64         | 4.43 ± 0.20 (+20)| 1.54 ± 0.07 (+16)    | 98.6 ± 0.7 (+4)        |
| 128        | 4.6 ± 0.23 (+24) | 1.58 ± 0.06 (+19)    | 100.4 ± 0.8 (+6)       |

Values in parenthesis are [%] decrease or increase (+). The results represented are the mean ± SEM of three independent experiments (n = 3) performed in triplicate (*p* < 0.05, represent significant difference compared with control).

were severely affected by increasing concentrations of ZnCl₂ followed by N-ZnO, but no such significant changes were observed in the case of M-ZnO treatment. The damage to the pigment except carotenoids was directly proportional to the concentration of the toxicant and became prominent after 8 μM and 32 μM of ZnCl₂ and N-ZnO exposure, respectively. Whereas, the carotenoid content increased significantly from 2–8 μM of ZnCl₂ and 16–32 μM of N-ZnO treatment, showing its antioxidant potential at low concentrations of Zn²⁺. In contrast, after treated with 2–128 μM of M-ZnO, little bit enhancement in both the photosynthetic pigments was observed only at the highest dose (128 μM). Photosynthetic parameters proved to be very useful for envisaging the toxicity profiles of heavy metals and metal NPs [24]. Heavy metals (Pb²⁺ and Cd²⁺) leads to dose-dependent (0, 0.5, 1, 2, 4, 6 and 8 mg/L) decline in photosynthetic pigment content and growth of unicellular cyanobacterium *Synechocystis* sp. PCC 6803 [25]. Approximately 20% and 80% growth inhibition occurs at 200 and 300 mg/L of ZnO NPs, respectively and more than 50% reduction in photosynthetic pigments in 300 mg/L ZnO NPs treated Arabidopsis plants was also reported [26]. These metals induced damages to photosynthetic pigments could be due to the replacement of magnesium ions from the tetrapyrrole ring of chlorophyll molecules, electron transport chain impairment, damages to enzymes associated with chlorophyll synthesis or might be due to the ROS mediated peroxidation of membrane lipids [27].

Further, the enhanced level of superoxide and total peroxide radicals in *N. muscorum* after treated with different dose (2–128 μM) of ZnCl₂, N-ZnO and M-ZnO are shown in Figures 2 and 3, respectively. In the case of ZnCl₂ treatment, superoxide and peroxide level increased gradually by 12–121% and 18–132%, respectively in a dose-dependent manner up to 128 μM. A similar trend of increment in superoxide (5–98%) and peroxide (7–106%) radicals were also observed up to 128 μM of N-ZnO. But no such significant ROS formation (superoxide and peroxide) were observed in the case of M-ZnO exposure might be due to the insignificant dissolution of M-ZnO into their potential at low concentrations of Zn²⁺.
toxicological mechanisms of environmental NPs are facilitated by oxidative stress caused by ROS generation. Present results are supported by [29], who showed that significant amount of ROS could be produced in the cells even they are exposed to insignificant amounts of ZnO or CuO NPs.

Figure 4 elucidates the lipid peroxidation estimated as MDA content in N. muscorum exposed to 2–128 μM of ZnCl₂, N-ZnO, and M-ZnO for 72 h. Exposure of ZnCl₂ results in the enhanced accumulation (14–97%) of MDA up to 128 μM, depicting significant lipid peroxidation at higher concentrations. Whereas, after treated with N-ZnO, peroxidation of lipids increased gradually (4–72%) in a dose-dependent manner up to 128 μM. In contrast, treatment of M-ZnO results in less/insignificant peroxidation of lipid up to 64 μM. The lipid peroxidation under heavy metal stress as a marker of oxidative stress is a well-known phenomenon [30]. The enhanced level of lipid peroxidation in the N. muscorum treated with high dose of ZnCl₂ and N-ZnO depicted that their excess accumulation triggered the production of ROS, which caused the oxidative damage to the plasma membrane. These results are in accordance with [31], who showed that the amount of ROS was found to be much higher in metal oxide NPs suspensions compared to their bulk formulations.

As a result of this ROS generation, oxidative stress takes place into the cells, which accelerates the superoxide dismutase (SOD) and peroxidase (POD) activity in N. muscorum exposed to different concentration (2–128 μM) of ZnCl₂, N-ZnO and M-ZnO (Figures 5 and 6). The cells under ZnCl₂ stress showed enhanced SOD (26–78%) and POD (30–89%) activity up to 16 μM, after that sharp decline in both the scavenging activity was observed upto 128 μM, depicting severe toxicity of ZnCl₂ at higher concentrations, which ultimately results in cell death. While, under N-ZnO stress, both SOD (9–85%) and POD (15–93%) activity was increased in a dose-dependent manner up to 64 μM. In contrast, M-ZnO exposure results in little bit enhancement in both SOD and POD activity only at highest concentrations (64 and 128 μM).

Defense mechanisms get accelerated to combat the deleterious effects of reactive oxygen species in various
of enhancement in SOD activity in the leaves of Arabidopsis thaliana and in marine microalgae Tetraselmis gracilis following Cd treatment was stated [35]. Besides the enhancement of SOD activity, POD activity has also been found to be accelerated in various terrestrial and aquatic sp. exposed to Zn and Al, a similar pattern of results was also reported in the present study [36].

Besides, these enzymatic antioxidants some non-enzymatic antioxidants are also accumulated against the resulting oxidative stress. Figures 7 and 8 demonstrate the proline and total phenol accumulation in N. muscorum exposed to 2–128 μM of ZnCl₂, N-ZnO, and M-ZnO for
72 h. Exposure of ZnCl₂ results in proline and total phenol accumulation by 18–58% and 20–62%, respectively, in a dose-dependent manner up to 16 μM, after that decline in both the non-enzymatic antioxidants was observed up to 128 μM by 25–70% and 21–65%, respectively. The decline in proline and total phenol accumulation at higher concentrations might be due to the overproduction of free radicals, which might lead to the cell death. While in the case of N-ZnO, the proline and total phenol content increased by 6–76% and 8–87% in a dose-dependent manner up to 64 μM. It is known that proline and many phenolic metabolites are induced under abiotic as well as biotic stress and accumulated in tissue after different environmental stresses. Many plants exposed to drought or a high salt content in the soil known to accumulate proline to combat the effects of various stresses [37]. Besides, acting as an osmolyte and important metal chelator, these metabolites also scavenges the free radicals to provide protection against the oxidative stress-mediated cell damage [38]. In the same way, the decline in stress correlates with the quantity of total free phenols because these phenolics exhibit properties of antioxidant due to the availability of free phenolic hydrogen [39]. These metabolites are accumulated under stress condition might be to participate in ROS scavenging through antioxidative enzymes, like H₂O₂ scavenging in assistance of peroxidases [40].

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