Archives of Environmental Protection
Vol. 50 no. 2 pp. 85–92

PL ISSN 2083-4772
DOI.10.24425/aep.2024.150555

Effects of copper and zinc on *Microcystic aeruginosa* growth and microcystins production

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Keywords: Cyanobacteria bloom; Heavy metals; Microcystins; *Microcystic aeruginosa*

Abstract: The cyanobacteria bloom is one of typical manifestations of eutrophication, yet the effects of heavy metals entering water on cyanobacteria bloom remain unclear. In the present study, the effects of copper and zinc ions on the growth of *Microcystic aeruginosa* (*M. aeruginosa*) and the production of microcystins (MCs) were investigated. The results showed that a Cu^{2+} concentration of 0.02 mg/L stimulated the growth of *M. aeruginosa*, while growth inhibition occurred at a Cu^{2+} concentration of 0.1 mg/L. The maximum value of MC-LR (167.74 μg/L) occurred at a Cu^{2+} concentration of 0.02 mg/L. In contrast, a Zn^{2+} concentration of 0.1 mg/L stimulated the growth of *M. aeruginosa,* whereas growth inhibition was observed at a Zn^{2+} concentration of 0.5 mg/L. The maximum MC-LR value of 130 μg/L appeared under control conditions. Moreover, the production of MC-LR increased as the growth of *M. aeruginosa* was inhibited with a Cu^{2+} concentration of 0.1 mg/L, whereas the production of MC-LR decreased as the growth of *M. aeruginosa* was stimulated with a Zn^{2+} concentration of 0.1 mg/L, compared to their respective controls.

Introduction

In recent years, the occurrence of eutrophication in aquatic ecosystems has become a challenging environmental problem worldwide (Du et al. 2022, Brookes and Carey 2011). Eutrophication is a process characterized by nutrient overload in water bodies, leading to the excessive growth of plants and algae. This phenomenon often results in the development of unpleasant tastes and odors on the water surface, adversely affecting water supplies and recreational activities (Xu et al. 2021, Svircev et al. 2017). The eutrophication process has been accelerated by rapid industrialization and urbanization (Shen et al. 2018). Cyanobacterial blooms are one of the most typical manifestations of eutrophication (Krishnan et al. 2020, Kormas et al. 2011), They are prevalent in many lakes and reservoirs around the world, such as Lake Erie, in the US-Canada region (Newell et al. 2019), Lake Taihu in China (Huisman et al. 2018), and Salto Grande in Uruguay-Argentina (Gangi et al. 2019). Cyanobacteria blooms deteriorate aquatic ecosystems by depleting the oxygen needed by other organisms to live. Some species of cyanobacteria in these blooms even pose risks to human health by producing cyanotoxins known as microcystins (MCs) (Drobac et al. 2013, Bouron et al. 2015). MCs comprise a group of heptapeptide hepatotoxins with various forms, with Microcystin-LR (MC-LR) being the most toxic (Paerl and Otten 2013).

Cyanobacteria typically multiply and bloom in waters rich in phosphorus and nitrogen (Han et al. 2017). These nutrients are affected by various environmental factors, such as light, temperature, pH, dissolved oxygen, ultraviolet radiation and so on (Bucak et al. 2018, Paerl et al. 2011). Moreover, cyanobacteria are integral to the phytoplankton community in aquatic ecosystems, and trace metals are essential for their functioning (Facey et al. 2019). Many studies have demonstrated that metal ions, such as nickel (Ni), copper (Cu), cadmium (Cd), iron (Fe) and zinc (Zn), are necessary for the growth of cyanobacteria, followed by toxin production within a certain concentration rang (Dai et al. 2016, Admiraal et al. 1995). However, at higher concentrations, metal ions can inhibit cyanobacteria multiplication due to their toxic effects (Chakraborty et al. 2010).

The anthropogenic impacts on aquatic ecosystems frequently include industrial wastewater and domestic sewage. Consequently, heavy metals, organic contaminants, or mixed organic and inorganic pollutants commonly coexist in polluted waters (Polyak et al. 2013). In this context, understanding the effects of heavy metals pollution on cyanobacteria growth and cyanotoxin production is essential for addressing eutrophication. Therefore, the following research goals were formulated: (I) summarize the effects of copper and zinc on *M. aeruginosa* growth and cyanotoxin production, (II) determine superoxide dismutase (SOD) activity, catalase (CAT) activity, reactive oxygen species (ROS) activity, and malondialdehyde (MDA) content in *M. aeruginosa* under copper and zinc stress, (III) discuss the influence of colimitation on cyanobacterial bloom and MCs production in eutrophication associated with

Figure. 1. The equipment used for the experimental.

heavy metals pollution waters. These results will be helpful in paving the way for controlling cyanobacterial blooms in eutrophication associated with heavy metals pollution waters.

Materials and methods

Materials

The *M. aeruginosa* strain FACHB-912 was purchased from the Institute of Hydrobiology, Chinese Academy of Sciences. The strain was cultured in modified BG11 medium composed of 500 mg/L $NaNO_3$, 40 mg/L $K_2HPO_4.3H_2O$, 2.86 mg/L H_3BO_3, 1.81 mg/L $MnCl_2.4H_2O$, 6 mg/L $FeCl_3.6H_2O$, 75mg/L $MgSO_4.7H_2O$, 36 mg/L $CaCl_2.2H_2O$, 1 mg/L Edta Na_2, and 6 mg/L Citric acid. The cultures were grown in a series of 250-mL Erlenmeyer flasks and incubated in an illuminated incubator (GZX-80 , China) at 25±1°C, with light intensity of 22.5 μmol/m^2 /s) and at 12:12 h light–dark (L:D) photoperiod. The experimental equipment and flowchart are shown in Fig.1 and Fig. 2, respectively.

The effect of Cu^{2+} and Zn^{2+} on M. aeruginosa growth and MCs production

To assess the impact of initial Cu^{2+} and Zn^{2+} concentrations on *M. aeruginosa* growth and MCs production, 1.0-mL cultures of the strain were added to 100 mL of modified BG11 medium containing various concentrations of Cu^{2+} ($CuSO_4$) or Zn^{2+}

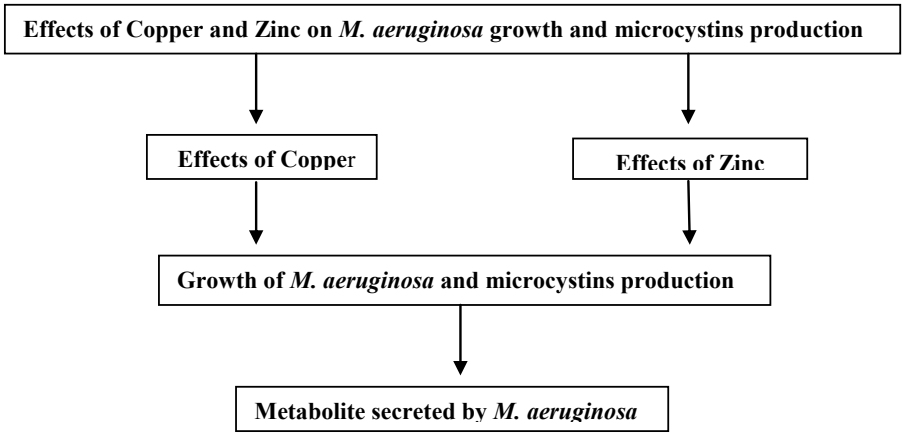

Figure. 2. The individual stages of research.

($ZnSO_4$) in a series of 250-mL Erlenmeyer flasks. The flasks were then placed in an incubator set at 25±1°C, with a light intensity of 22.5 μ mol/m^2 /s and a 12:12 h light–dark (L:D) photoperiod. Control flasks without Cu^{2+}or Zn^{2+} were incubated in parallel under the same conditions. At specific intervals, samples were withdrawn from the flasks to determine the algal density and MC-LR concentrations.

The effect of Cu^{2+} and Zn^{2+} concentration on SOD, CAT, MDA and ROS secreted by M. aeruginosa

To assess the effects of the initial Cu^{2+} and Zn^{2+} concentrations on the secretion of SOD, CAT, MDA, and ROS by *M. aeruginosa,* 1.0-mL cultures of the strain was added to 100 mL of modified BG11 medium containing various concentrations of Cu^{2+} or Zn^{2+} in a series of 250-mL Erlenmeyer flasks. The flasks were then placed in an incubator set at 25±1°C, with a light intensity of 22.5 μ mol/m^2 /s) and a 12:12 h light–dark (L:D) photoperiod. Control flasks without Cu^{2+}or Zn^{2+} were incubated in parallel under the same conditions. At the conclusion of the experiments, samples were withdrawn from the flasks, and the levels of SOD, CAT, MDA, and ROS secreted by *M. aeruginosa* were determined.

Analytical methods

The cultures of strain FACHB-912 were collected regularly during the experimental period (once every 4 days). Algal density was investigated by counting cells using an optical microscope. MC-LR concentrations were determined as follows: 9 mL of the culture were re-dissolved in deionized water and disrupted by three times of freezing (-70°C) and thawing (40°C) (Oberemm et al. 1999). The solution was passed through a 0.45-μm fiber filter, and the supernatant was collected and concentrated using a solid phase extraction cartridge (Cleanert C18 500 mg/ 6 mL cartridge, (Phenomenex and Agela, Tianjin, China). The MC-LR concentrations were analyzed using high performance liquid chromatograph (HPLC) with a 1mL solution. The HPLC (UltiMate 3000, Shenzhen ruisheng technology co. Ltd, China) was equipped with a reverse C18 column (5μm, 250mm×4.6 mm), the column temperature was maintained at 30 °C , and the mobile phase consisted of acetonitrile and 0.05 % trifluoroacetic acid (50:50 v/v) at a flow rate of 1 mL/min, and an injection amount of 10 μl.

The SOD activity, CAT activity and MDA content were all measured in triplicate using Enzyme-linked Immunosorbent Assay (ELISA) kits (Hefei Laier biotechnology co. Ltd, Hefei, China) according to the manufacturers' instructions. ROS activity was measured triplicate using an ELISA kit (Beyotime biotechnology co. Ltd, Shanghai, China) following the provided instruction.

Experimental replication setting and data analysis

All of the tests were carried out in triplicate, and the presented results are the averages of the triplicate measurements. Data were processed using Origin 8.5. The relationship between SOD, CAT, ROS and MDA were analyzed using SPSS software (Product and Service Solutions Statistical, 22.0) through Pearson correlation analysis. The significance was determined with a 2-tailed value of < 0.05, considered statistically significant.

Results

The effect of the initial Cu^{2+} and Zn^{2+}concentration on Growth of M. aeruginosa

The effect of the initial Cu^{2+} concentration (0.02, 0.05, 0.10 mg/L) on the growth of *M. aeruginosa* is shown in Fig. 3a. In comparison with the control, no clear differences in the lag period were observed when the initial Cu^{2+} concentration ranged from 0.02 to 0.1 mg/L. As the growth of *M. aeruginosa* reached the stationary phase, the cell densities were $6.72\times10^7$and 6.13×10^7 cells/mL at Cu^{2+} concentration of 0.02 and 0.10 mg/L, respectively, whereas that of the control was 6.44×10^7 cells/mL. Therefore, the growth of *M. aeruginosa* was promoted at Cu^{2+} concentration of 0.02 mg/L but was inhibited at Cu^{2+} concentration of 0.10 mg/L.

Fig. 3b shows the growth of *M. aeruginosa* at different concentrations of Zn^{2+}. Compared with the control, there was no significant difference in the curve patterns when the concentration of $Zn^{2+}$was 0.1 mg/L. However, the growth of *M. aeruginosa* was inhibited as the Zn^{2+}concentration increased from 0.1 to 0.5 mg/L. The maximal cell density was 6.7×10^7 at a Zn^{2+} concentration of 0.1 mg/L, while the minimal cell density was 5.5×10^7 at a Zn^{2+} concentration of 0.5 mg/L.

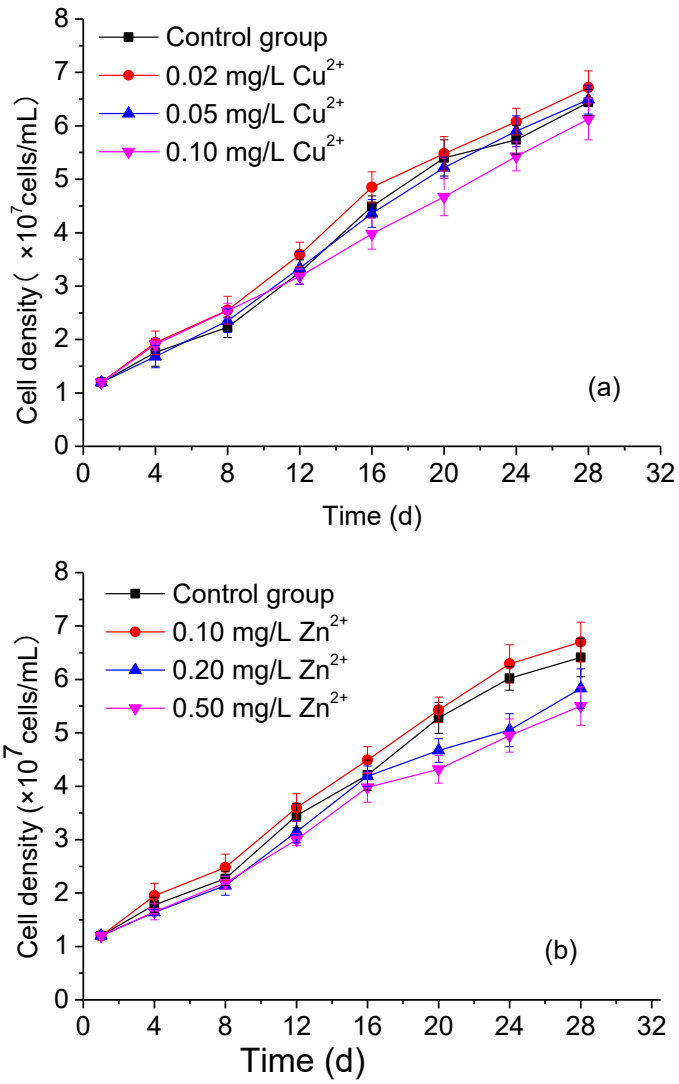

Figure. 3. The effects of the initial Cu^{2+} (a) and Zn^{2+} (b) concentrations on *M. aeruginosa* growth.

Table. 1. The effect of the initial Cu^{2+} (Zn^{2+}) concentration on MC-LR production by *M. aeruginosa*.

Cu^{2+} concentration (mg/L)	MC-LR (μg/L)	Zn^{2+} concentration (mg/L)	MC-LR (μg/L)
control	128.05±20.7	control	130.2± 12.5
0.02	167.74±16.6	0.1	97.77± 16.4
0. 05	101.05±17.8	0. 2	106.36± 15.4
0.1	136.23±17.5	0. 5	79.36± 11.0

The effect of the initial Cu^{2+} and Zn^{2+}concentration on MC-LR production by M. aeruginosa

The effect of the initial Cu^{2+} concentrations (0.02, 0.05, 0.10 mg/L) on MC-LR production by *M. aeruginosa* is shown in Table 1. It is evident that the maximum value of MC-LR (167.74 μg/L) occurred at a Cu^{2+} concentration of 0.02 mg/L, while the minimum value of MC-LR (101.05 μg/L) appeared at a Cu^{2+} concentration of 0.05 mg/L, which was lower than that of the control (128.05μg/L).

Table 1 also shows the effect of the initial Zn^{2+} concentrations (0.10, 0.20, 0.50 mg/L) on MC-LR production by *M. aeruginosa*. In comparison with the control, it is evident that the MC-LR contents decreased with Zn^{2+}concentrations ranging from 0.10 to 0.50 mg/L, respectively. The maximum value of MC-LR (130.2 μg/L) occurred in the control, while the minimum value of MC-LR (79.36 μg/L) appeared at a Zn^{2+} concentration of 0.5 mg/L.

The effect of the initial Cu^{2+} and Zn^{2+} concentration on SOD, CAT, MDA and ROS secreted by M. aeruginosa

The effects of initial Cu^{2+} concentrations (0.02, 0.05, 0.10 mg/L) and Zn^{2+} concentrations (0.10, 0.20, 0.50 mg/L) on SOD, CAT, MDA and ROS secreted by *M. aeruginosa* on the 25th and 28th days are show in Fig. 4-7. The levels of SOD and CAT observed under copper stress on the 28th day were higher than those secreted on the 25th day, while the levels of ROS and MDA observed under copper stress on the 28th day were lower than those secreted on the 25th day. Similarly, the levels of SOD

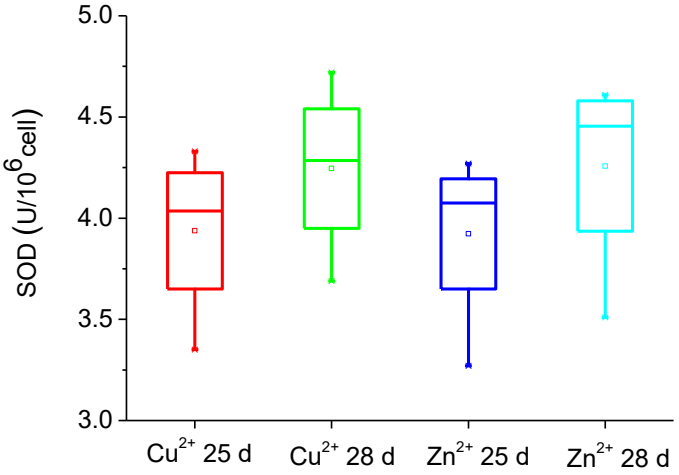

Figure. 4. The effects of the initial Cu^{2+} and Zn^{2+} concentrations on SOD production by *M. aeruginosa*.

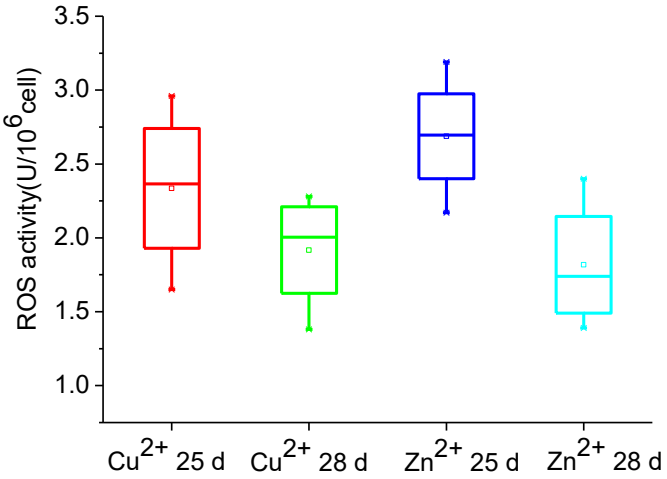

Figure. 6. The effects of the initial Cu^{2+} and Zn^{2+} concentrations on ROS production by *M. aeruginosa*.

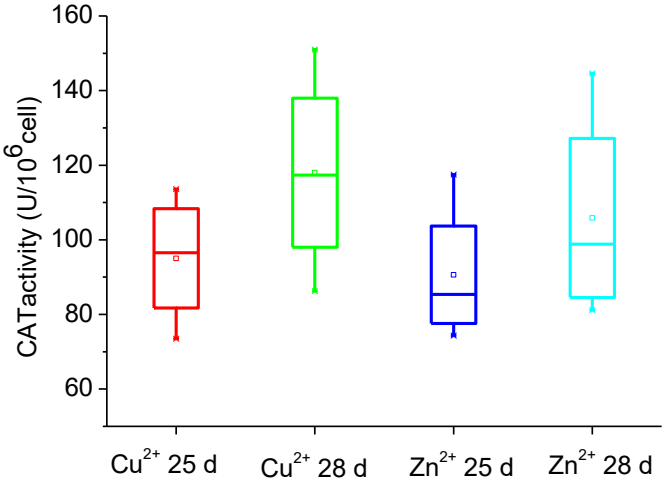

Figure. 5. The effects of the initial Cu^{2+} and Zn^{2+} concentrations on CAT production by *M. aeruginosa*.

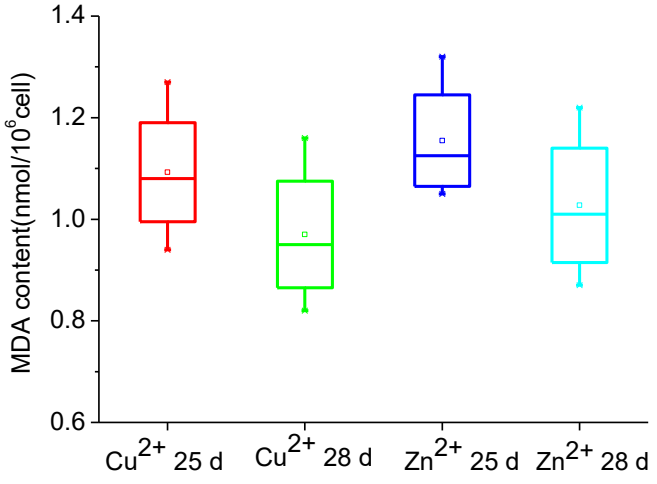

Figure. 7. The effects of the initial Cu^{2+} and Zn^{2+} concentrations on MDA production by *M. aeruginosa*.

Table. 2. The effect of the initial Cu^{2+} and Zn^{2+} concentration on SOD, CAT, MDA and ROS secreted by *M. aeruginosa.*

NO	Factors	SOD activity (U/10^6 cell)	CAT activity (U/10^6 cell)	ROS activity (U/10^6 cell)	MDA kontent (nmol/10^6 cell)
1	Cu^{2+} (control, 25 d)	4.12	89.95	2.52	1.05
2	Cu^{2+} (0.02 mg/L, 25 d)	4.33	113.58	1.65	0.94
3	Cu^{2+} (0.05 mg/L, 25 d)	3.95	103.15	2.21	1.11
4	Cu^{2+} (0.1 mg/L, 25 d)	3.35	73.48	2.96	1.27
5	Cu^{2+} (control, 28 d)	4.36	109.77	2.14	0.91
6	Cu^{2+} (0.02 mg/L, 28 d)	4.72	151.03	1.38	0.82
7	Cu^{2+} (0.05 mg/L, 28 d)	4.21	124.93	1.87	0.99
8	Cu^{2+} (0.1 mg/L, 28 d)	3.69	86.28	2.28	1.16
9	Zn^{2+} (control, 25 d)	4.12	89.95	2.76	1.05
10	Zn^{2+} (0.1 mg/L, 25 d)	4.27	117.47	2.17	1.08
11	Zn^{2+} (0.2 mg/L, 25 d)	4.03	80.8	2.63	1.17
12	Zn^{2+} (0.5 mg/L, 25 d)	3.27	74.31	3.19	1.32
13	Zn^{2+} (control, 28 d)	4.36	87.92	1.59	0.96
14	Zn^{2+} (0.1 mg/L, 28 d)	4.61	144.63	1.39	0.87
15	Zn^{2+} (0.2 mg/L, 28 d)	4.55	109.77	1.89	1.06
16	Zn^{2+} (0.5 mg/L, 28 d)	3.51	81.13	2.4	1.22

and CAT observed under zinc stress on the 28th day were higher than those secreted on the 25th day, while the levels of ROS and MDA observed under copper stress on the 28th day were lower than those secreted on the 25th day.

SOD, CAT, MDA and ROS secreted by *M. aeruginosa* under 16 different conditions are shown in Table 2. It is evident from Fig.8 that SOD, CAT, MDA and ROS secreted by *M. aeruginosa* under the condition of Cu^{2+} and Zn^{2+} could be clustered into 3 classes, I(1, 9, 8, 13, 11, 16, 4, 12); II (5, 15, 2, 10, 3, 7); and III (6, 14). These clusters imply the effect on SOD, CAT, MDA and ROS secreted by *M. aeruginosa* under the condition of Cu^{2+}(0.02 mg/L, 28 d) and Zn^{2+} (0.1 mg/L, 28

Table. 3. The correlation between SOD, CAT, ROS and MDA.

		SOD	CAT	ROS	MDA
SOD	Pearson correlation	1	0.808**	-0.832**	-0.922**
	Sig.(2-tailed)		0.000	0.000	0.000
	Number	16	16	16	16
CAT	Pearson correlation	0.808**	1	-0.816**	-0.833**
	Sig.(2-tailed)	0.000		0.000	0.000
	Number	16	16	16	16
ROS	Pearson correlation	-0.832**	-0.816**	1	0.862**
	Sig.(2-tailed)	0.000	0.000		0.000
	Number	16	16	16	16
MDA	Pearson correlation	-0.922**	-0.833**	0.862**	1
	Sig.(2-tailed)	0.000	0.000	0.000	
	Number	16	16	16	16

** Correlation is significant at the 0.01 level (2-tailed)

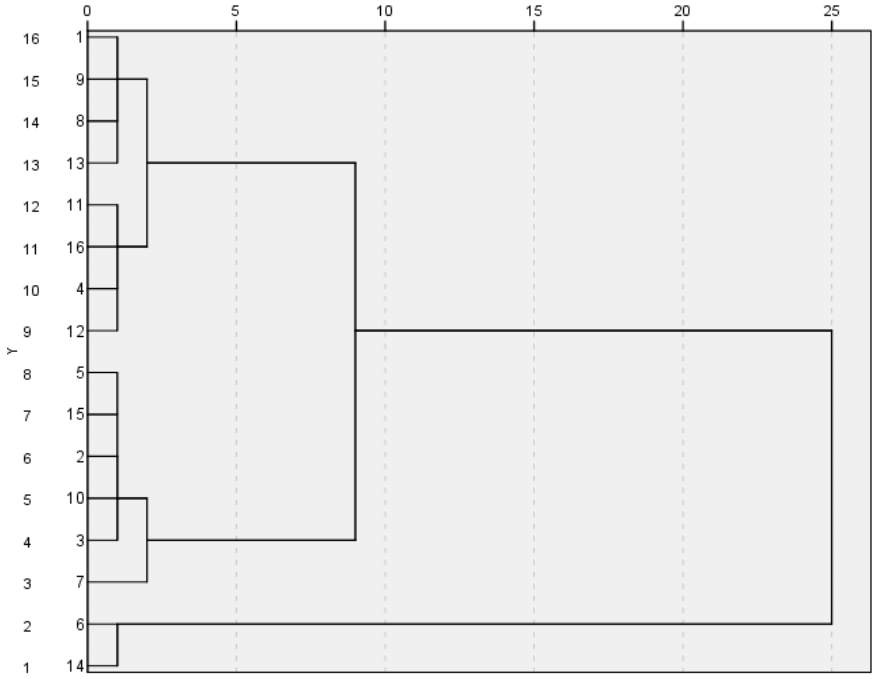

Figure. 8. Dendrogram of Hierarchical Cluster Analysis.

d) greatly differed from SOD, CAT, MDA and ROS secreted by *M. aeruginosa* under other conditions. Table 3 illustrates the relationship between SOD, CAT, ROS and MDA. The correlation coefficients of 0.808 and 0.862 indicate a very high positive correlation between SOD and CAT, MDA and ROS, respectively. Meanwhile, the relationships between SOD, ROS, SOD and MDA, CAT and ROS, and CAT and MDA show high negative correlations, with correlation coefficients of -0.832, -0.922, -0.816, and -0.833, respectively.+

Discussion

The importance of trace metals for algal proliferation and MCs synthesis has been widely studied in previous laboratory investigation (Cavet et al. 2003, Zhou et al. 2013). In the present study, a Cu^{2+} concentration of 0.02 mg/L stimulated the growth of *M. aeruginos*a, whereas the growth of the species was inhibited at a Cu^{2+} concentration of 0.1 mg/L. These results were similar to the observations by Tsai (2015), indicating that the minimum Cu^{2+} concentration required to inhibit *M. aeruginosa* growth was 0.160 mg/L. Low concentrations of Cu^{2+} are essential for *M. aeruginos*a growth due to metal cofactors in enzymatic activities, while high concentrations can be highly toxic to *M. aeruginos*a growth (Bishop et al. 2015).

Our results demonstrated that the optimum growth (the cell densities) and maximum value of MC-LR occurred at a Cu^{2+} concentration of 0.02 mg/L, simultaneously. However, the minimum value of MC-LR appeared at a Cu^{2+} concentration of 0.05 mg/L. These findings differ from observations by Chen (2020), who found that intracellular MCs synthesis of *M. aeruginos*a PCC 7806 increased with both 0.5 μM and 3 μM copper treatments. This discrepancy could be explained by the inhibition of *M. aeruginos*a's photosynthetic capacity in our study, which may be related to the biosynthesis of intracellular MC productions at high Cu^{2+} concentrations (Ao et al. 2019). Moreover, the MC-LR value at a Cu^{2+} concentration of 0.1 mg/L was higher than that at a concentration of 0.05 mg/L, probably because that the extracellular MC concentration gradually increased with elevated Cu^{2+} concentrations and prolonged exposure time (Tsai et al., 2015).

The present study also demonstrated that a Zn^{2+} concentration of 0.1 mg/L stimulated the growth of *M. aeruginosa*, whereas the growth of the species was inhibited at a Zn^{2+} concentration of 0.5 mg/L, with the cell densities being lower than those of the control. These results are similar to the observations by Zhou (2019), who found that the optimal concentration of zinc for algal growth was 0.05 mg/L, while growth inhibition of *M. aeruginosa* began at a Zn^{2+} concentration of 1 mg/L. A possible explanation for these findings is that low concentrations of Zn^{2+} serve as essential micronutrients for the growth of *M. aeruginosa,* whereas higher concentrations of Zn^{2+} negatively affect electron transport, subsequently inhibiting cell growth (Zhou et al. 2018).

The maximum value of MC-LR appeared at the control, However, the minimum value of MC-LR occurred at a Zn^{2+} concentration of 0.5 mg/L. In comparison with the control, MC-LR concentration decreased with the addition of Zn^{2+}. These findings contrast with observations by Ao (2019), who found that MC content of dry algal cells was restrained in Zn-deficient conditions. This disparity suggests that intracellular MC concentration increased in the absence of zinc stress, as metal ion starvation has been reported to increase microcystin synthesis (Sevilla et al. 2008).

The optimum growth (the cell densities) and maximum MC-LR values under copper stress were higher than those observed under zinc stress (Fig.3 and Table 1). This indicates that the highest levels of SOD and CAT occurred under copper stress on the 28th day, while the highest levels of ROS and MDA (an indicator of ROS formation) appeared under zinc stress on the 25th day (Fig 4-7). Higher levels of SOD (and CAT) are beneficial for ROS removal and reducing the risk of damaging

cellular macromolecules (Martínez-Ruiz & Martínez-Jerónimo 2016), suggesting that the oxidative damage to cellular components caused by Zn^{2+} is higher than that caused by Cu^{2+} .

Conclusions

In the present study, the roles of Cu^{2+} and Zn^{2+} in the proliferation of *M. aeruginosa* and the synthesis of MCs were investigated. The results show that the growth of *M. aeruginos*a was stimulated by exposure to low concentrations of Cu^{2+} or Zn^{2+}, while high concentration of Cu^{2+} or Zn^{2+} inhibited its growth. The inhibition concentrations for *M. aeruginosa* growth were determined to be 0.1 mg/L for Cu^{2+} and 0.5 mg/L for Zn^{2+}. The maximum and the minimum value of MC-LR appeared at Cu^{2+} concentrations of 0.02 and 0.05 mg/L, respectively. However, MC-LR production decreased with different concentration treatments of Zn^{2+} compared to the control. The maximum and the minimum values of MC-LR appeared at the control and 0.5 mg/L, respectively. The growth of *M. aeruginosa* was not related to MC-LR production under copper or zinc stress. Further research is needed to elucidate the detailed mechanisms by which Cu^{2+} and Zn^{2+} affect the synthesis of MC-LR in *M. aeruginosa.*

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