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Effect Of Tetracycline on The Growth and Function of Autogenic Nitrogen-Fixing Bacteria

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***Abstract*—The misuse of Tetracycline in agriculture has led to a significant increase and accumulation of residues in the soil environment. Tetracycline (TC) is one of the most commonly detected tetracycline antibiotics in recent years. To study the specific impact of antibiotics on soil microorganisms, this experiment used nitrogen-fixing bacteria as a biological model and co-cultured them with different concentrations of tetracycline medium. The co-culture products were then measured to record the growth, nitrogen fixation activity, morphological changes, and oxidative stress of the nitrogen-fixing bacteria. The results showed that tetracycline inhibited the growth, nitrogen fixation activity, and enzyme activity of nitrogen-fixing bacteria, with 2 mg/L of tetracycline and higher concentrations exhibiting stronger physiological and biochemical inhibitory effects on nitrogen-fixing bacteria. Our study indicates that Tetracycline are toxic to nitrogen-fixing microorganisms.**

***Index Terms*—Antibiotics, Biological nitrogen fixation, Nitrogen-fixing bacteria, Toxicity effects**

I. INTRODUCTION

Since the 1990s, antibiotics have received special attention due to the extensive use and consumption of antibiotics in the environment, the rapid expansion of antibiotic resistance and the emergence of multiple drug-resistant pathogenic fungi [1]. Urban, agricultural and industrial wastewater are the main sources of antibiotics and their by-products in the environment [2]. Because of their antibacterial and persistence, the traditional biological wastewater treatment process often has a limited effect on their degradation [3]. The reported that the bacteriostatic properties of residual antibiotics will inevitably affect the metabolic process of microorganisms [4]. Biological nitrogen fixation is a unique physiologica function of nitrogen-fixing microorganisms, which is carried out under the catalysis of nitrogenase [5]. Therefore, it is particularly important to explore the effects of different concentrations of antibiotics on nitrogen-fixing bacteria [6].

Biological nitrogen fixation refers to the conversion of free nitrogen in the air into bioavailable nitrogen by microorganisms [7].The nitrogen in the soil is converted into available nitrogen by nitrogenase [8].Authigenic nitrogen-fixing bacteria are widely distributed in soil and water in nature.The proliferation of antibiotics will affect the reproduction and function of microorganisms because of their bacteriostatic properties [9]. As a kind of antibiotics, Tetracycline is widely used in disease treatment and prevention. As substances circulate into the environment, it has certain toxicity to animals [10], plants [11], microorganisms [12] and human beings [13].

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The purpose of this study was to test the specific effects of Tetracycline on soil azotobacter bacteria [14] and explore the specific differences [15] caused by it at the genetic level. High concentration of Tetracycline significantly inhibited the growth activity of azotobacter bacteria, and the nitrogen fixing activity of azotobacter bacteria was significantly inhibited, which provided basic data for the development of environmental concentration standards of antibiotics through scanning electron microscopy observation and oxidative stress experiment. It has theoretical and practical significance.

II. EXPERIMENTAL

Materials preparation

Sucrose, mannitol, yeast, peptone, Sodium chloride, anhydrous calcium chloride, ferric sodium ethylenediamine tetraacetate, sodium molybdate dihydrate, crystalline magnesium sulfate, potassium nitrate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium hydroxide, Tetracycline.

The culture medium prepared in this experiment is used to culture azotobacter brown. According to the culture medium prepared in 1 L, 20 g sucrose, 20 g mannitol, 1 g yeast, 1 g peptone, 0.1 g sodium chloride, 1 g magnesium sulfate heptahydrate, 0.2 g dipotassium phosphate, 0.6 g potassium dihydrogen phosphate, 0.5 g potassium nitrate, and 1 g sodium chloride. Anhydrous calcium chloride 0.1g, ethylenediamine tetraacetate ferric sodium salt 0.13g, sodium molybdate dihydrate 0.05g.Weigh the above drug, add deionized water to the required scale line, use a magnetic stirr to fully dissolve the drug in the deionized water, measure 50 mL of the prepared solution to 100 mL conical bottle, and put it in an autoclave for sterilization.

Bacterial growth experiment

Tetracycline was dissolved in sodium hydroxide solution and prepared into TC solution with concentrations of 0.5 mg/L、1 mg/L、2 mg/L、5 mg/L、10 mg/L, and 50 μL was added into 50 ml culture medium. At this time, the concentration of SMX in culture medium was 0.5 mg/L、1 mg/L、2 mg/L、5 mg/L、10 mg/L.A. vinelandii solution was incubated at 30 ℃ and 120 rpm, then theabsorbance (OD600) was measured on a UV－vis spectrophotometer at 12h intervals up to 72 h. At 72 h, the bacterial suspension (50 μL) was serially diluted and inoculated onto solid medium. After static incuba-tion at 30 ◦C for another 36 h, the CFU values were counted by a colony counter (Scan-300, Wisdom Shanghai Instrument Co, China).

Nitrogen fixation activity of A. vinelandii

Relative nitrogenase activity

The relative nitrogenase activity of A. vinelandii was determined by the ^{15}N isotope dilution method. ^{15}N-KNO_3 (0.1 g, Adamas Co,Shanghai, China) was added to 1 L of liquid medium and transferred to Erlenmeyer flasks (50 mL per flask). After 72 h incubation, 1 mL ofbacterial suspension was collected and lyophilized. Then, 1.000 mg of the uniformly mixed sample was weighed and added to a tin foil. The air in the tin foil was squeezed out and the $^{15}N/^{14}N$ ratio was assayed by isotope ratio mass spectrometer (IRMS, isoprime visION, Elementar,Germany).

Absolute nitrogenase activity

The absolute nitrogenase activity of nitrogen-fixing bacteria was determined using the acetylene reduction assay (ARA) (Wang et al., 2017a). The culture medium samples, after 72 h of cultivation (10.0 mL each), were transferred to headspace sampling vials, sealed with a cap, and evacuated under vacuum. After confirming the seal, the reaction gas and protective gas were introduced in the ratio of $Ar:O_2:C_2H_2$ = 6:1:1. Once filled, the samples were placed in a constant temperature shaker for further cultivation for 2 hours. Gas samples (1.00 mL) were analyzed using a gas chromatograph (GC9720, Zhejiang Fuli Analytical Instruments Co., Ltd., China), and the ethylene production rate per unit time and mass of the sample was calculated (Yan et al., 2020).

Ultrastructural changes

For scanning electron microscopy (SEM) observation, A. vinelandii cells were collected at 72 h by centrifugation at 3000 rpm for 10 min.The residues were suspended and fixed with 2.5 % glutaraldehyde for 3 h. The cells were washed twice by PBS and a series of methanol solution,and finally soaked in pure methanol overnight. The cells were centri- fuged and air-dried in a fume hood. A. vinelandii cells were coated with gold (JFC 1600, JEOL, Japan) and then imaged under SEM (JSM-7500, JEOL, Japan).

Membrane leakage, energy metabolism and oxidative stress

These indicators of A. vinelandii were measured by commercial kits of protein, lactate dehydrogenase (LDH), $Ca^{2+}Mg^{2+}$-ATPase, ATP content, total superoxide dismutase (SOD), glutathione (GSH) and malondial- dehyde (MDA) from Nanjing Jiancheng Bioengineering Institute. The bacterial suspensions were collected at 72 h postexposure and sampled following the recommended protocols from the manufacturer.

III. RESULTS AND DISCUSSION

Growth of A. vinelandii.

This experiment designed six concentration groups, with three parallel samples in each group. The CFU values determined by the dilution plate method in Figure 1a at a concentration of 0.5 mg/L were 102.40% of the control group, also due to the toxic stimulatory effect. Starting from a concentration of 1 mg/L, the number of nitrogen-fixing bacteria colonies per unit area decreased as the TC concentration increased. At 10 mg/L, the measured value was only 29.13% of the control group, indicating that adding TC inhibited the growth and reproduction of nitrogen-fixing bacteria, with a dose-dependent effect; the higher the TC concentration added, the fewer the surviving nitrogen-fixing bacterial colonies.

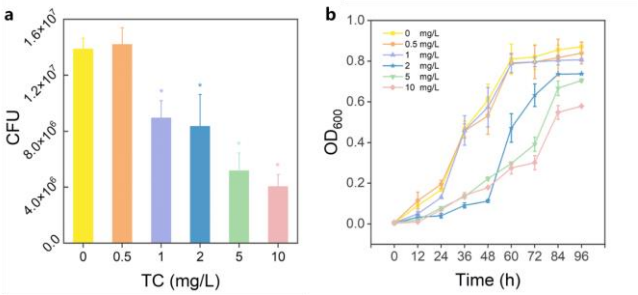

Figure 1 Effects of different concentrations of tetracycline (TC) on the growth of *A.vinelandii*. (a) CFU value, colony forming units, bacterial colony count; (b) Growth curves. *Indicates a significant difference between the control and experimental groups, p<0.05.

Figure 1b similarly confirms this point, showing no significant changes in absorbance of nitrogen-fixing bacteria at 0 h and 12 h under different tetracycline concentrations. However, after 24 h of cultivation, differences in absorbance began to appear, along with an increase in growth rate. When the TC concentration was 0.5 mg/L, the growth curve measured did not differ much from the control group. However, when the concentration exceeded 2 mg/L, the absorbance significantly decreased compared to the control group, and the slope of the curve also decreased, indicating a slowdown in growth rate during the cultivation process. Similarly, when the absorbance was measured after 96 h of cultivation, the absorbance value of the 10 mg/L group was only 66.38% of the control group's absorbance value, consistent with the CFU measurement results. The addition of TC affects the growth activity of nitrogen-fixing bacteria.

Nitrogen fixation of A. vinelandii

Figure 2a shows the determination of nitrogenase activity in nitrogen-fixing bacteria using acetylene reduction, with TC addition concentration at 0.5 mg/L. The detected ethylene production rate was 96.88% of that in the control group; as the addition concentration increased, the acetylene production rate decreased, indicating that after adding tetracycline, the nitrogen fixation rate of nitrogen-fixing bacteria slowed down, and their nitrogen-fixing activity declined. The experimental results show that as the TC concentration increases, the overall nitrogen-fixing activity of nitrogen-fixing bacteria is inhibited.

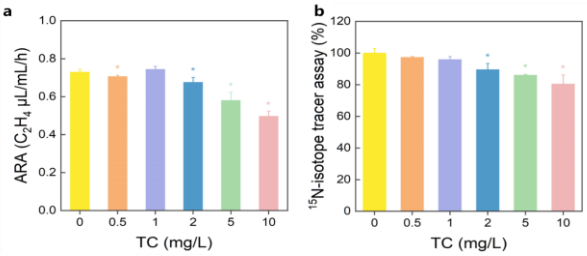

Figure 2 Effects of different concentrations of tetracycline (TC) on nitrogen-fixing arectivity of *A.vinelandii*. (a) Acetylene reduction analysis; (b) Isotope dilution analysis. *Indicates a significant difference between the control and experimental groups, p<0.05.

Figure 2b shows the use of isotope dilution to determine the nitrogen-fixing ability of nitrogen-fixing bacteria using the 15N/14N ratio. At 0.5 mg/L, the ratio is 3.77% lower than that of the control group. When the maximum addition concentration reaches 10 mg/L, the $^{15}N/^{14}N$ ratio is 80.40% higher than that of the control group. As the TC addition concentration increases, the fixed N value decreases, consistent with the results obtained from acetylene reduction. This indicates that the nitrogen-fixing activity of Vickersia bacteria is influenced by TC, and the inhibitory effect becomes more pronounced as the TC addition concentration increases.

Characteristics of ultrastructure changes of A. vinelandii.

The morphological changes of nitrogen-fixing bacteria under SEM are shown in the figure. The control group remains intact (Figure 3a). When the TC concentration is 0.5 mg/L, folds appear on the surface of Vickers nitrogen-fixing bacteria (Figure 3b), as indicated by the arrow pointing to a different part from the control group. When the TC concentration is 5 mg/L, the number of cells under the same magnification lens significantly decreases (Figure 3c), and some cells show obvious signs of indentation and shrinkage damage.

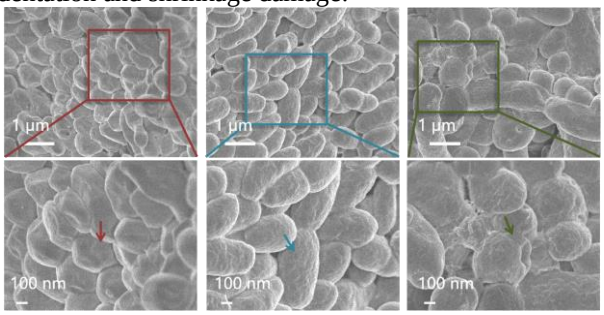

Figure 3 Scanning electron microscopy (SEM) images of the morphological characteristics of *A.vinelandii.* at different concentrations of TC. (a) control group; (b) 0.5 mg/L TC; (c) 5 mg/L TC. The arrow points to the damaged cells.

Effects of tetracycline on energy metabolism and oxidative stress in V. nitrogen-fixing bacteria.

The H_2O_2 and CAT levels of Vorticella nitrogen-fixing bacteria exposed to TC showed a concentration-dependent increase (Figure 4a). At 0.5 mg/L, the nitrogen-fixing bacteria had not completely lost their function, with a small increase in measured values, indicating that the organisms were still responding to stress. At 10 mg/L, induced oxidative stress was most severe (Figure 4c), with MDA levels increasing by 320.24%, GSH levels by 246%, SOD levels by 207.84%, and LDH levels by 166.56%. Under high concentrations, the nitrogen-fixing bacteria exhibited stress responses after being subjected to certain stresses.

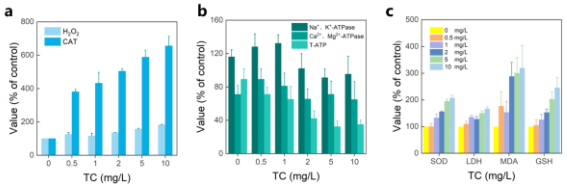

Figure 4 Effects of TC on oxidative stress and energy metabolism in *A.vinelandii.* (a) H2O2, hydrogen peroxide, CAT, catalase; (b) energy metabolism; (c) SOD, Superoxide dismutase; LDH, lactate dehydrogenase; MDA, malondialdehyde; GSH Glutathione.

As the concentration continued to rise, Vorticella nitrogen-fixing bacteria could no longer withstand the induced toxicity, leading to cellular weakness and inability to resist external stress through their own stress response, resulting in massive cell death. The decrease in $Ca^{2+}Mg^{2+}$-ATP enzyme activity (Figure 4b) explains why the nitrogen-fixing activity of nitrogen-fixing bacteria decreased after adding TC, and excessive addition of TC caused cell damage, naturally affecting the nitrogen-fixing activity of the bacteria.

IV. CONCLUSION

In recent years, the distribution and effects of human and veterinary antibiotics in the environment have been a topic of in-depth research. Studies have shown that antibiotic exposure can alter the structure and function of microbial communities in soil and sediments.This experimental systemically studied the impact of tetracycline (TC) on nitrogen-fixing bacteria. The experiments revealed that at lower concentrations, TC does not significantly inhibit the morphology, growth status, activity, or nitrogenase activity of nitrogen-fixing bacteria. However, when the concentration is higher, it begins to negatively affect the physiological characteristics such as growth and reproduction of nitrogen-fixing bacteria. At extremely high concentrations, it can lead to cell damage and death, causing negative impacts on the physiological and biochemical aspects of nitrogen-fixing bacteria. The results of oxidative stress clearly demonstrate this point.In the future, attention should be paid to the emission concentration of antibiotics in order to reduce the impact on the ecological environment.

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