He *et al. Annals of Microbiology* (2024) 74:2
https://doi.org/10.1186/s13213-023-01747-2

Annals of Microbiology

UNIVERSITÀ DEGLI STUDI DI MILANO

ORIGINAL ARTICLE

Open Access

Remediation of copper-contaminated soils and growth enhancement of Pakchoi (B. *chinensis* L.) via biofertilizers composed of new *Bacillus amyloliquefaciens* SYNU1

Haisheng He[1], Sijia Bao[2], Yannan Wu[2] and Deli Tong[2*]

Abstract

Background Excessive copper contaminants are harmful to soil, microbes, plants and humans and can be remediated by biosorption. Applying biofertilizers to remediate copper-contaminated soil is an environmentally friendly way.

Results In this study, we identified a new strain, *Bacillus amyloliquefaciens* SYNU1, based on morphological, biochemical, physiological and phylogenetic analyses. It has been cultured on Luria–Bertani (LB) medium and absorbs soluble Cu^{2+} from pH 3.5–5 and 25–40 °C at Cu^{2+} concentrations of 100 mg L^{-1}. The results showed that the maximum adsorption capacity of copper by strain SYNU1 is 53.09% w/w. Furthermore, microbe fertilizers were made based on strain SYNU1, these fertilizers were allowed to ferment for 15 days, and they were used for remediation of copper-contaminated soil and growth tests of Pakchoi (*B. chinensis* L) in pot experiments. The results showed that the growth of Pakchoi planted in copper-contaminated soil at concentrations ranging from 50 to 200 mg kg^{-1} was inhibited, and its growth indices, such as plant height, fresh weight and dry weight, decreased significantly with increasing copper concentration. Compared with the control, the increases in plant height, fresh weight and dry weight of Pakchoi treated with biofertilizer were 10.37, 65.3 and 67.78%, respectively, indicating that biofertilizer could significantly promote the growth of Pakchoi.

Conclusions *Bacillus amyloliquefaciens* SYNU1 is useful for the bioremediation of Cu^{2+}-contaminated soil in Northeast China.

Keywords Biofertilizer, *Brassica campestris* ssp. *chinensis* L, Copper, *Bacillus amyloliquefaciens* SYNU1

Introduction

Moderate amount of copper is an essential micronutrient for the growth and development of all biological organisms, and it exists in many oxidation states and plays key roles in numerous physiological processes, such as photosynthesis, respiration, hormone perception, cell wall metabolism and oxidative stress protection in plants (Yruela 2005; Pilon et al. 2006). At the cellular level, copper also plays a signalling role in transcription and protein trafficking machinery, oxidative phosphorylation and iron mobilization (Puig et al. 2007). Thus, plants need copper to maintain normal growth. When copper iron is not available, plants develop topical symptoms, such as apical meristem necrosis and young leaf wilting (Liu et al. 2013; Hansda et al. 2015; Leng et al. 2015; Choinska-Pulit et al. 2018).

*Correspondence:
Deli Tong
dltong@synu.edu.cn
[1] Experimental Teaching Centre, Shenyang Normal University, Liaoning Province, Shenyang 110034, China
[2] College of Life Science, Shenyang Normal University, Liaoning Province, Shenyang 110034, China

However, high concentrations of copper have high toxicity in plants, which inhibits plant growth and impairs important physiological processes such as photosynthesis and respiration. High levels of copper are also highly toxic in humans and can accumulate in the brain, skin, liver, heart muscle and pancreas, causing great harm to human health (Patsikka et al. 2002; Yruela 2005; Pilon et al. 2006; Puig et al. 2007; Stern et al. 2007; Yruela 2009; Marques et al., 2019; Hossain et al. 2020; Gong et al. 2021).

During the development of agriculture, excessive use of Cu-based pesticides and fertilizers and wastewater irrigation have resulted in high copper concentrations in agricultural land, which have decreased the land quality, reduced the crop yield and caused great economic losses. Copper ions are enriched and transferred through the food chain, posing a great threat to environmental safety and human health. Therefore, it is very important and necessary to remediate copper-contaminated soil. Traditional methods require complicated equipment, are expensive and easily produce secondary pollution. In contrast, bioremediation has become a hotspot in the remediation of heavy metal pollution due to its advantages of environmental friendliness, low cost, wide source of materials, short remediation period and easy operation (Özdemir et al. 2013; Verma et al. 2013; Han et al. 2020; Yin et al. 2021; Li et al., 2022; Wang et al., 2022).

Applying biofertilizers to remediate heavy metal-contaminated soil is an environmentally friendly, cost-effective and sustainable option. Biofertilizers contain living microorganisms and organic matter, which can convert the available metal ions in soil into stable metal ions, reducing their biological availability. In addition, applying biofertilizers can improve soil nutrients and provide nutrients for plant growth. Remediating heavy metal-contaminated soil by using biofertilizer not only has the advantages of easy operation, environmental friendliness and no secondary pollution but can also promote the growth of plants and improve the disease resistance of plants (Gajdos et al. 2012; Bhardwaj et al. 2014; Derakhshan Nejad et al. 2018; Wang et al. 2019). Therefore, in this study, we hypothesized that biofertilizer could be effective in immobilizing soil Cu and alleviating its toxic effect on *Brassica campestris* ssp. *chinensis* L. This effect is related to biofertilizers reducing the copper content of the bioavailable state in soil, improving *B. chinensis* L antioxidant enzyme gene expression and improving antioxidant contents.

The purpose of this study was to evaluate the effectiveness of biofertilizers on the remediation of copper-contaminated soil. The antioxidant enzyme gene expression and antioxidant content in *B. chinensis* L. were evaluated. The information provided in this paper is useful for providing a theoretical basis and technical support for safe remediation strategies for soil heavy metal pollution.

Materials and methods

Molecular identification of the SYNU1 strain

The SYNU1 strain was provided by the Key Biological Laboratory of Shenyang Normal University. Initial cultures were revitalized from glycerol stocks stored at − 80 °C by streaking onto LB agar plates and incubating at 37 °C for 24 h. The cultures were regularly transferred to fresh LB medium every 2 to 4 weeks to maintain viability and ensure purity. Before each experiment, a single colony was picked from the fresh LB agar plate to start a pre-culture. After incubation at 160 rpm and 37 °C for 18 h, the culture medium was centrifuged at 1200 rpm for 2 min using the high-speed refrigerated centrifuge (Eppendorf 5427R, Germany), and the SYNU1 cell precipitate was obtained. A bacterial genomic DNA extraction kit (OMEGA, China) was used to extract genomic DNA. Universal primers for the *16S rDNA* gene (27f and 1492r) and *gyrB* gene (UP1 and UP2r) were used for amplification of genomic DNA by polymerase chain reaction (PCR) (Singh et al. 2013). PCR was carried out by Bio-Rad CFX96 PCR system (USA) with 10 μL Premix Taq, 0.5 μL 27f and 0.5 μL 1492r, 8 μL ddH_2O and 1 μL DNA as template.

The PCR procedure of the *16S rDNA* gene was conducted as follows: 95 °C for 5 min, then 30 cycles of 95 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR procedure of the *gyrB* gene was conducted as follows: 95 °C for 5 min, then 25 cycles of 93 °C for 60 s, annealing at 50 °C for 60 s and extension at 72 °C for 70 s, and a final extension at 72 °C for 10 min. The PCR products were analysed by 0.8% (w/v) agarose gel electrophoresis. The target gene fragments were cut, purified and recovered using a PCR Cleanup Kit (OMEGA, China). The recovered products were connected to the pMD18-T vector with size 2692 bp (Takara Bio Inc., Japan) and transformed into *E. coli DH5α* competent cells. Then, the competent cells were cultured for 1 h, and 100 μL of culture was applied to an LB plate containing ampicillin and incubated at 37 °C for 12 ~ 24 h. Colonies were selected for colony PCR and electrophoresis analysis in 1% (w/v) agarose gels. The colonies showing positive electrophoresis bands were sequenced by Thermo Fisher Scientific (China) Co., Ltd. The nucleotide sequences of the *16S rDNA* gene and *gyrB* gene of the SYNU1 strain were uploaded to the Ezbiocloud (https://www.ezbiocloud.net/) and NCBI (https://www.ncbi.nlm.nih.gov/) databases for comparison. Homologous sequences were obtained, and a phylogenetic tree was constructed by Mega11 software (Tamura et al. 2021).

The SYNU1 strain was stained by the Gram method and observed by optical microscopy under an oil-immersion lens. The SYNU1 strain was subjected to a starch hydrolysis test, methyl red test, v-p test, indole test, sugar fermentation test, urease test, nitrate reduction test and citrate utilization test.

Liquid kinetic adsorption mechanism of the SYNU1 strain on copper

Biomass preparation

The SYNU1 strain was grown in an LB medium (LB medium: sucrose 2.0 g L^{-1}, yeast 4.0 g L^{-1}, peptone 6.0 g L^{-1}, $MgSO_4$ 5.0 g L^{-1}). The aseptic operation was carried out on a super-clean work table, and 100 mL of LB liquid medium was filled into a 250-mL triangular flask. The SYNU1 bacterial solution was inoculated at an inoculation rate of 1% (V:V). After incubation at 160 rpm by AEOLUS™ IS-18CA/IS-18A Stackable CO_2 Incubator Shaker (USA) and 37 °C for 24 h, the cultures were centrifuged at 10,000 rpm and 4 °C for 15 min and washed triply with distilled deionized water to wash off adhering debris and culture medium. For determination of the cell dry weight of bacteria, the bacteria were oven-dried at 80 °C until a constant weight was attained (Li et al. 2017).

Copper stock solution

1.965 g $CuSO_4.5H_2O$ solid was dissolved in 50 mL sterile deionized water at a concentration of 10 g L^{-1}, sterilized by filtration and stored at 4 °C. Other copper concentrations were obtained by diluting the stock solution with sterile deionized water.

Unless otherwise indicated, 20 mL of copper solution (100 mg L^{-1}) and 2 mL of bacterial solution (1 g L^{-1}) were shaken in a 50-mL conical flask at 150 rpm and 35 °C for 4 h in all experiments. The effects of adsorption time (1–12 h), adsorption temperature (25–45 °C), copper solution pH (3 ~ 5), adsorbent dosage (1–2.5 g L^{-1}) and copper concentration (40–200 mg L^{-1}) were determined (Andreazza et al. 2010).

Preparation of biofertilizers and copper-contaminated soils

The SYNU1 strain was grown in LB liquid medium at 180 rpm and 37℃ for 48 h. Chicken manure, rapeseed meal, rice bran, soybean meal, fish bone meal, peat charcoal and seaweed powder were mixed evenly in a proportion of 50:10:10:10:10:5:5, and 60% tap water and 5% (v/w) fermentation broth of SYNU1 strain were added. Then, the mixture was fermented at room temperature for 15 days, and the biofertilizer was obtained.

The soil samples (0 ~ 20 cm) used for laboratory experiments were collected from the test site of the Shenyang Normal University (41° 54′ 33″ N, 123° 25′ 37″ E), Liaoning Province, China. Soil samples were homogenized, air-dried and sieved to 2 mm. Copper-contaminated soils were prepared with increasing concentrations of copper ranging from 0, 50, 100, 150 and 200 mg kg^{-1} by using $CuSO_4.5H_2O$. The metal salt was dissolved in distilled water, sprayed on the soil sample and mixed thoroughly (Haneef et al. 2014). Then, copper-treated soil samples were incubated in a pot and kept for 30 days. Before planting, the actual copper concentrations in the incubated soils were determined using atomic absorption spectroscopy (AAS).

Pot experiment of B. chinensis L

Pot experiments were conducted with the incubated soil samples to evaluate the effect of biofertilizers on the remediation of copper-contaminated soil and the growth of *B. chinensis* L. Each pot was filled with 300 g of soil thoroughly mixed with 3% (w/w) biofertilizers. For each treatment, three replicates were kept. Control pots were kept for both treatments without copper. Fifteen-day-old healthy seedlings of *B. chinensis* L. was transplanted to the experimental pots. Plants were grown under natural environmental conditions for 30 days. The growth indicators of plants were recorded in each pot.

Soil samples and plant tissue analysis for copper

Representative samples of initial soil and biofertilizer were collected, air-dried and ground to 1 mm before chemical analysis. The pH (solid: deionized water = 1:5 w/v) of the soil samples was measured. The control soil and contaminated soil were analysed for copper concentration by atomic absorption spectroscopy.

The plants were harvested after 40 days of growth, and their growth indices and heavy metal contents in different parts were measured. The stem length was recorded, and the aboveground parts of the plants were washed very carefully with deionized water four times. Plant tissues were oven-dried at 80 °C, and the constant dry weights were recorded. The dried plant materials were ground to less than 1 mm for copper analysis. The plants were analysed for copper concentration by atomic absorption spectroscopy (UV-3600i Plus Spectrophotometer, Japan).

Gene expression analysis by real-time PCR

The leaves of *B. chinensis* L. were harvested after 40 days of growth, and leaves with treatment and without treatment were also collected for expression pattern detection. All the materials were first pulverized with liquid nitrogen and stored in a − 80 °C refrigerator. Then, total RNA was extracted from leaves by using the RNAprep pure plant plus Kit (Tiangen Biotech Co., Ltd). The concentration and quality of RNA were determined by a spectrophotometer and detected by agarose gel electrophoresis

(Wang 2014). First-strand cDNA was synthesized from 2 mg of total RNA with MMLV reverse transcriptase and random hexamer primers (Takara) according to the manufacturer's instructions. The cDNA samples were used for real-time PCR with primers designed for the selected genes, and *BcActin* was used as a housekeeping gene (Table 1). The PCR was performed in a 20 mL reaction mixture containing 200 nM of each primer, 1 × SYBR Green PCR Master Mix (Takara, Dalian, China), and approximately 30 ng cDNA. Real-time RT–PCR was performed using the Bio-Rad CFX96 system (Bio-Rad, Hercules, CA, USA). The reactions were carried out as follows: 3 min at 95 °C for denaturation, 10 s at 94 °C, 20 s at 60 °C and 30 s at 72 °C for amplification for 45 cycles. The relative abundance of transcripts was calculated according to the software instructions in Bio-Rad CFX96 Manager. The specificity of each primer pair was verified by determining the melting curve at the end of each run and sequencing the amplified bands from gel electrophoresis.

Table 1 Primers for PCR of antioxidant enzymes genes in *B. chinensis* L

Genes	Primer sequence (5′–3′)	Amplification length (bp)
BcCuZnSOD	Forward: AGCAGTGAGGGTGTTAAGGG Reverse: GGTAGACATGCAACCGTTGG	150
BcAPX	Forward: AAGGTCTTCTTCAGCTCCCC Reverse: GAAAGCTTCAAGTGGGCCTC	130
BcCAT1	Forward: AAGCTTGCCAACTTCGACAG Reverse: CCTGGAGCTCTGAGGAAGTC	134
BcGR1	Forward: GCCAAGCACATTTTGATCGC Reverse: CTACCGCACGCTTAGGAAAC	121
BcActin	Forward: GCTTACGTCGCTCTTGACTACG Reverse: GATGGTGATGACTTGTCCATCAG	

Results

Identification of isolate SYNU1 based on phylogenetic, morphological and biochemical characterization

The strain SYNU1 is a rod-shaped bacterium observed under an oil-immersion lens (Fig. 1A). A biochemical test was further employed to analyse the characteristics of this bacterium. The results clearly showed that the bacteria are an aerobic and gram-positive strain. The catalase test, citrate solution test, nitrate reduction test, V-P test, hydrolysis of gelatine test and starch hydrolysis test were positive, whereas the urease test and indole test were negative (Table 2). The bacterium can ferment the sugars glucose, maltose, starch and sucrose to produce acid. The 16S rRNA sequence of the SYNU1 strain was aligned to those available in the NCBI database. The alignment results showed that the isolated strain SYNU1 was closely related to *Bacillus subtilis*, *Bacillus siamensis* and *Bacillus amyloliquefaciens*, with an identity greater than 99%. A specific species cannot

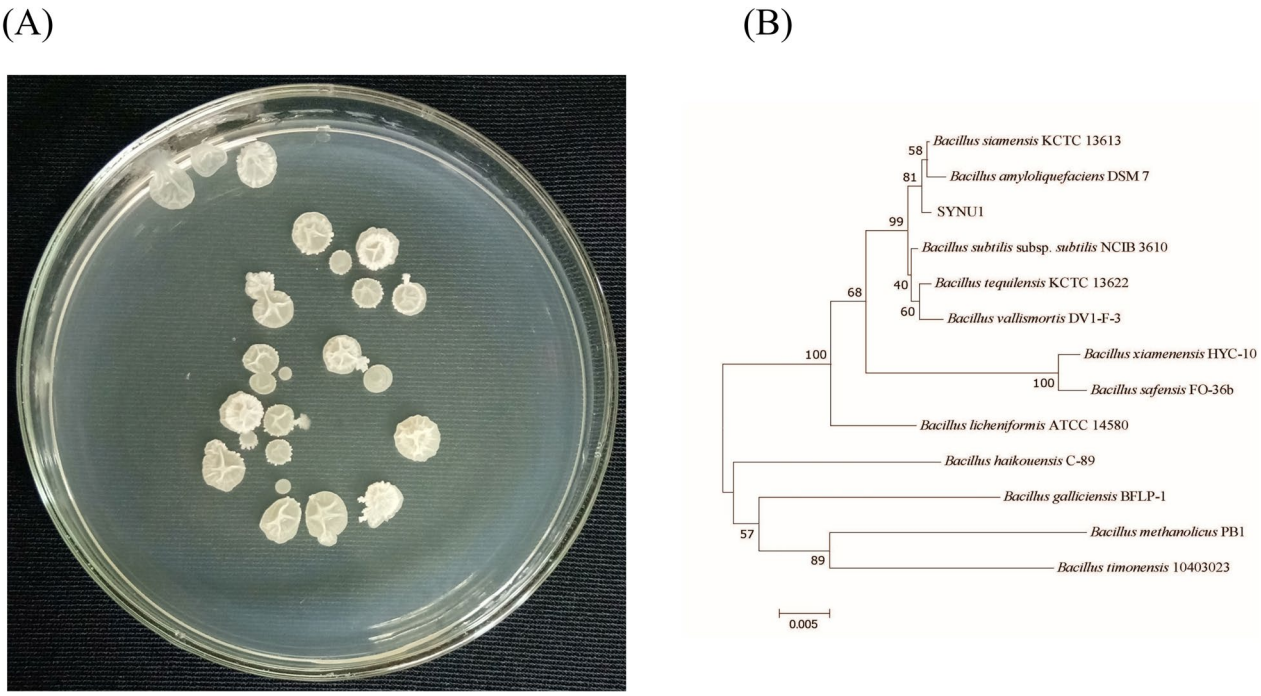

Fig. 1 SYNU1 strain morphology and phylogenetic tree based on the sequences of the *gyrB* gene sequences

Table 2 Some physiological and biochemical characteristics of SYNU1 strain

Test items	Results	Test items	Results
Gram stain	+	Hydrolysis of gelatine	
Starch hydrolysis	+	Citrate solution	+
Methyl red	–	Nitrate reduction	+
Catalase	+	Indole test	–
V-P test	+	Urease	–

be accurately identified by 16S rRNA gene sequences only. Complete sequences of the 1.2-kb gyrB fragments of B. amyloliquefaciens SYNU1 were cloned. Then, we spliced together the sequences of the 16S rRNA and gyrB genes of the SYNU1 strain and constructed a phylogenetic tree. The results showed that the SYNU1 strain and *Bacillus amyloliquefaciens* were clustered in the same group (Fig. 1B).

Effect of biosorption factors on Cu.$^{2+}$ biosorption by strain SYNU1

The effect of pH on the biosorption capacity of Cu(II) by strain SYNU1 is shown in Fig. 2A. It showed that upon increasing the pH from 3 to 5, the biosorption capacity increased first and then stabilized. The effect of different temperatures on Cu^{2+} biosorption by strain SYNU1 is shown in Fig. 2B, revealing that the temperature had no significant influence Cu^{2+} uptake. The adsorption capacity of copper by the SYNU1 strain at various initial Cu^{2+} concentrations shows that with an increase in the initial Cu^{2+} concentration from 40 to 200 mg L^{-1}, the biosorption efficiency decreases. A lower initial metal concentration resulted in a high adsorption rate. With increasing adsorbent biomass, the adsorption rate tends to rise first and then fall. The adsorption at different adsorbent dosages shows that the biosorption capability of Cu^{2+} by strain SYNU1 increases with increasing adsorbent dosage. The adsorption capacity of copper by strain SYNU1 was 53.09% when the adsorbent biomass was 2.0 g L^{-1}.

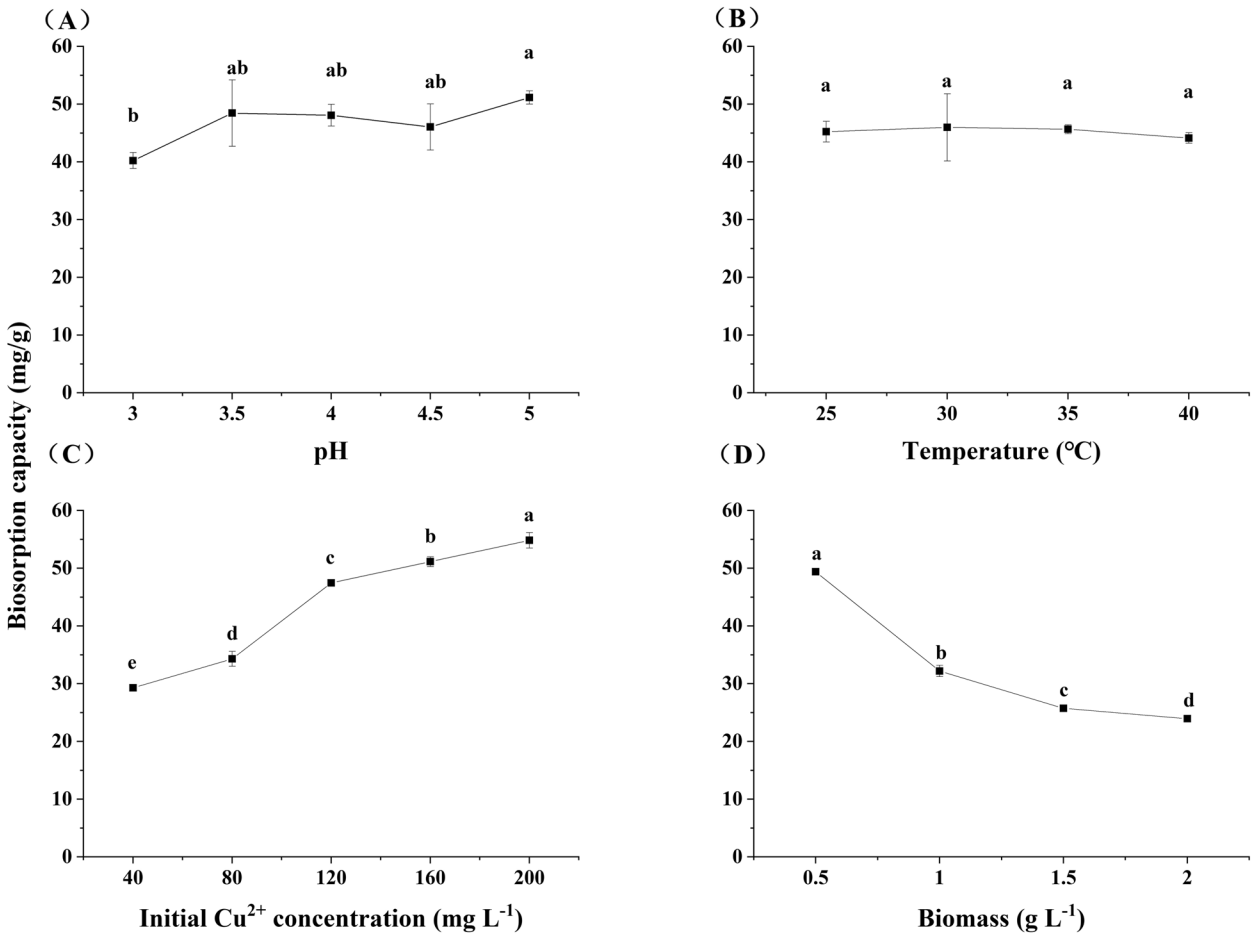

Fig. 2 The adsorption rate of copper by the SYNU1 strain under different adsorption conditions

Biofertilizer preparation

During biofertilizer fermentation, microbes decomposed the organic matter and released heat. In this study, the biofertilizer temperature increased rapidly during composting, peaked on the 5th day, and then decreased gradually with the maturation of biofertilizer (Fig. 3A). The colour of the biofertilizer was initially greyish brown. Because the humus content rose gradually during composting, the colour of the biofertilizer became dark brown (Fig. 3B). The odour of biofertilizers during preparation was pungent, which smelled strongly of ammonia.

Effect of biofertilizer on growth of B. chinensis L under copper stress

In this study, *B. chinensis* L. inoculated and not inoculated with biofertilizer showed significant differences in phenotypic traits under different copper concentrations (Fig. 4). The growth parameters, such as plant height, plant fresh and dry weight were significantly different among inoculated and non-inoculated biofertilizer at every level of copper treatment (Fig. 5), indicating that high copper concentrations could inhibit crop growth and decrease crop yield. When *B. chinensis* L was treated with different copper concentrations, the biomass of the treatment groups with biofertilizer was significantly higher than that without biofertilizer, indicating that biofertilizer can not only promote the growth of *B. chinensis* L but also alleviate the stress effect on *B. chinensis* L. We treated copper-contaminated soil with microbial fertilizer for 30 days, and the extractable Cu^{2+} content of all soil samples was extracted with 0.1 mol L^{-1} HCl. The results showed that there was no significant difference in the content of soil extractable Cu^{2+} between the treatments with biofertilizer and without biofertilizer when the copper concentration exceeded 100 mg kg^{-1}.

Expression level of antioxidant enzyme gene in B. chinensis L

Real-time PCR analysis showed that the expression of *CuZnSOD* increased with increasing copper concentration (Fig. 6). The expression of the *CuZnSOD* gene in *B. chinensis* L treated with biofertilizer was significantly lower than that without biofertilizer, which indicated that biofertilizer could reduce the toxicity of copper and alleviate copper stress on *B. chinensis* L. The expression of the *BcCAT1* gene in *B. chinensis* L. increased when treated with biofertilizer but decreased when treated without biofertilizer with increasing copper concentration. The expression patterns of the *BcAPX* gene and *BcGR* gene in *B. chinensis* L. treated with biofertilizer

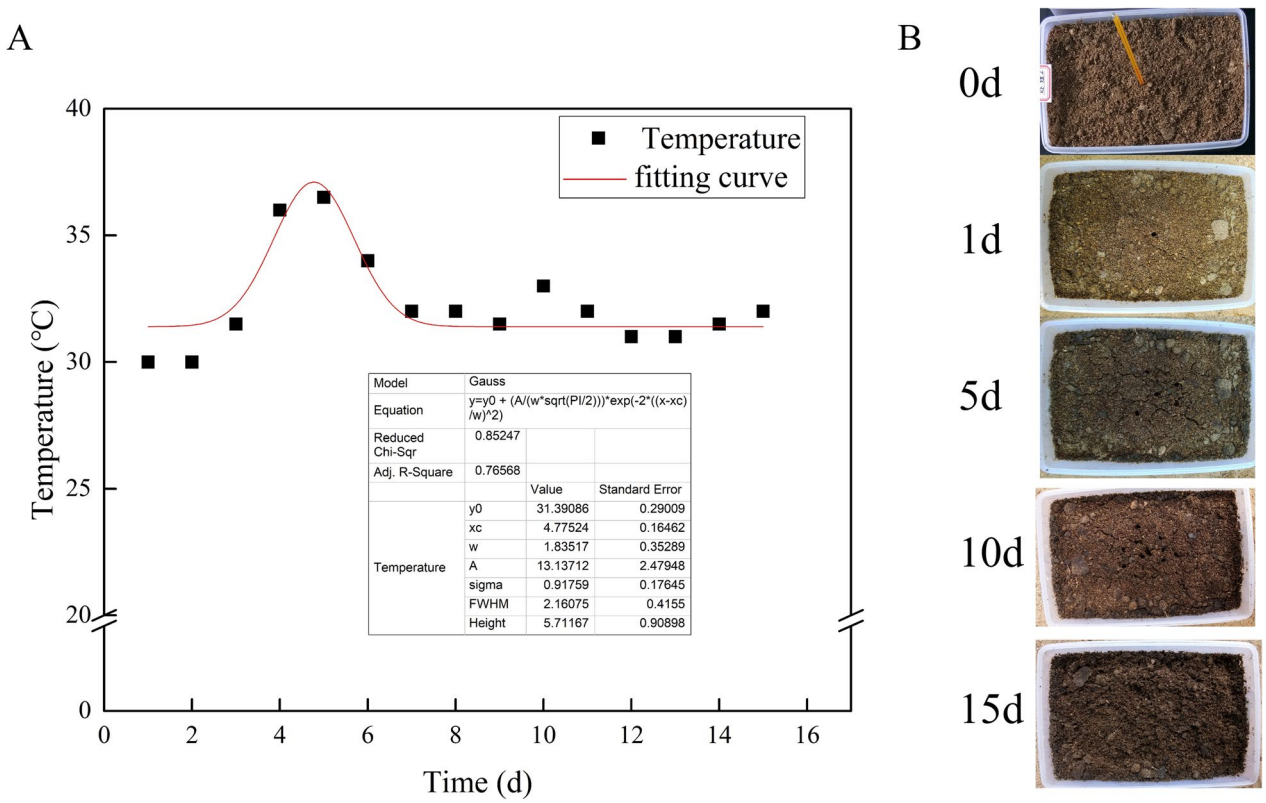

Fig. 3 Changes in fermentation temperature and biofertilizer colour

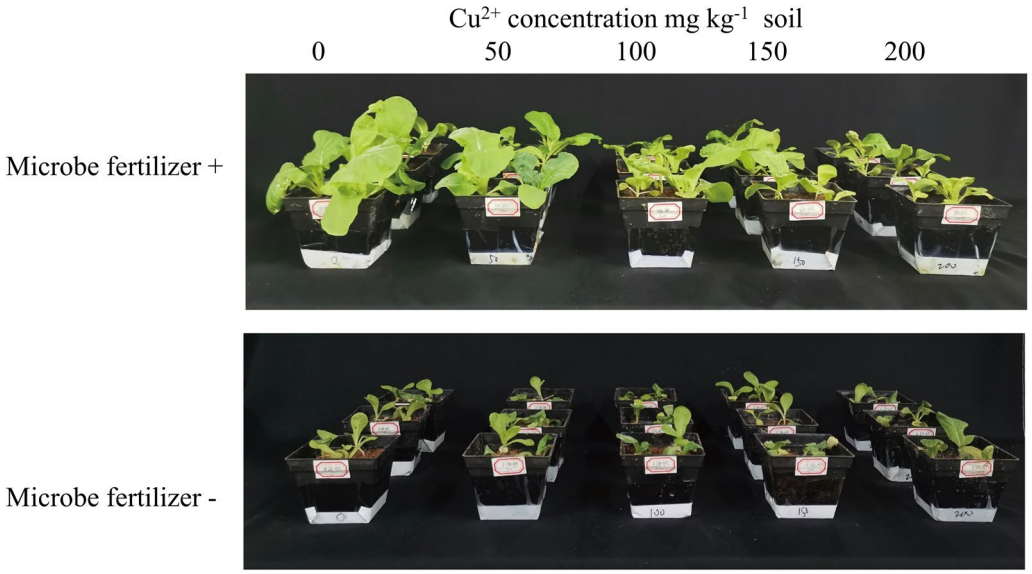

Fig. 4 Effect of biofertilizer on the growth of *B. chinensis* L. under different copper concentrations

and without biofertilizer were similar (Fig. 6), and their expression levels decreased with increasing copper concentration. The leaves of *B. chinensis* L. used in this study were collected after 30 days of growth in copper-contaminated soil, which is different from previous studies. Under long-term stress, plants do not have enough energy to synthesize multiple antioxidant enzymes at the same time, and only one of the more efficient types can be synthesized to continuously remove ROS.

Discussion

Microbe is useful for removing heavy metals and organic contaminants from soil and water (Tang et al. 2021). 16S rRNA gene sequencing is a widely practised technique for bacterial identification, but it has limitations for members of closely related species (Fox et al. 1992; Yamamoto and Harayama 1995). Some protein-coding genes, such as *rpoB* (Kim et al. 1999), *gyrB* (Yamamoto and Harayama 1995) and *gyrA* (Chun and Bae 2000), have been concluded to be useful for the identification of closely related taxa. The *gyrB* gene was used to identify the *Bacillus subtilis* group, which comprises eight closely related species with an identity range of 98.1–99.8% in 16S rRNA gene sequences, and the results showed that the range of *gyrB* gene sequence similarities among the eight type strains was 75.4–95.0% (Wang et al. 2007). This result indicated that the *gyrB* gene sequences provide higher resolution than 16S rRNA gene sequences (Fig. 1B).

The adsorption mechanism of heavy metals by microorganisms is a complex biological process that is affected by pH, temperature, adsorption time, metal concentration and adsorbent biomass. The analysis of those factors is important for evaluating the biosorption potential of biomaterials. This result was because higher concentrations of ionic copper was toxic to the bacterium and inhibited the normal growth and metabolism of the *Bacillus amyloliquefaciens* SYNU1, this was similar to previous studies (Peng et al. 2019). Furthermore, the transformation of Cu into a more stable form by microorganisms cannot be ignored (Sun et al. 2023). In this study, the temperature pattern was similar as that in previous studies (Chang and Yang 2009; Tsai et al. 2007) (Fig. 2). Among biocontrol agents, *B. amyloliquefaciens* is considered safe and to have high potential because it is ubiquitous in nature and exhibits high thermal tolerance by forming resistant spores. Generally, the main targets of *Bacillus* species biocontrol agents are powdery mildew and grey mould diseases (Tanaka et al. 2017). Because *B. amyloliquefaciens* has a strong inhibitory effect on *Botrytis cinerea* and *Podosphaera fusca*, *B. amyloliquefaciens* also has a significant effect on promoting plant growth and is often used to promote the growth of many crops, such as tomato, rice, cucumber and other crops. pH is an important factor that affects the solution chemistry of metals and the activity of the functional groups on the surface of microbial cells. It was established that a pH range between 3 and 6 was conducive to biosorption (Gadd and Rome 1988). Studies at pH values higher than 5 were not conducted because insoluble copper hydroxides precipitated and restricted true biosorption studies. It was established that temperature influences the biosorption process to a lesser extent within the range of 20–35℃ (Veglio and Beolchini 1997).

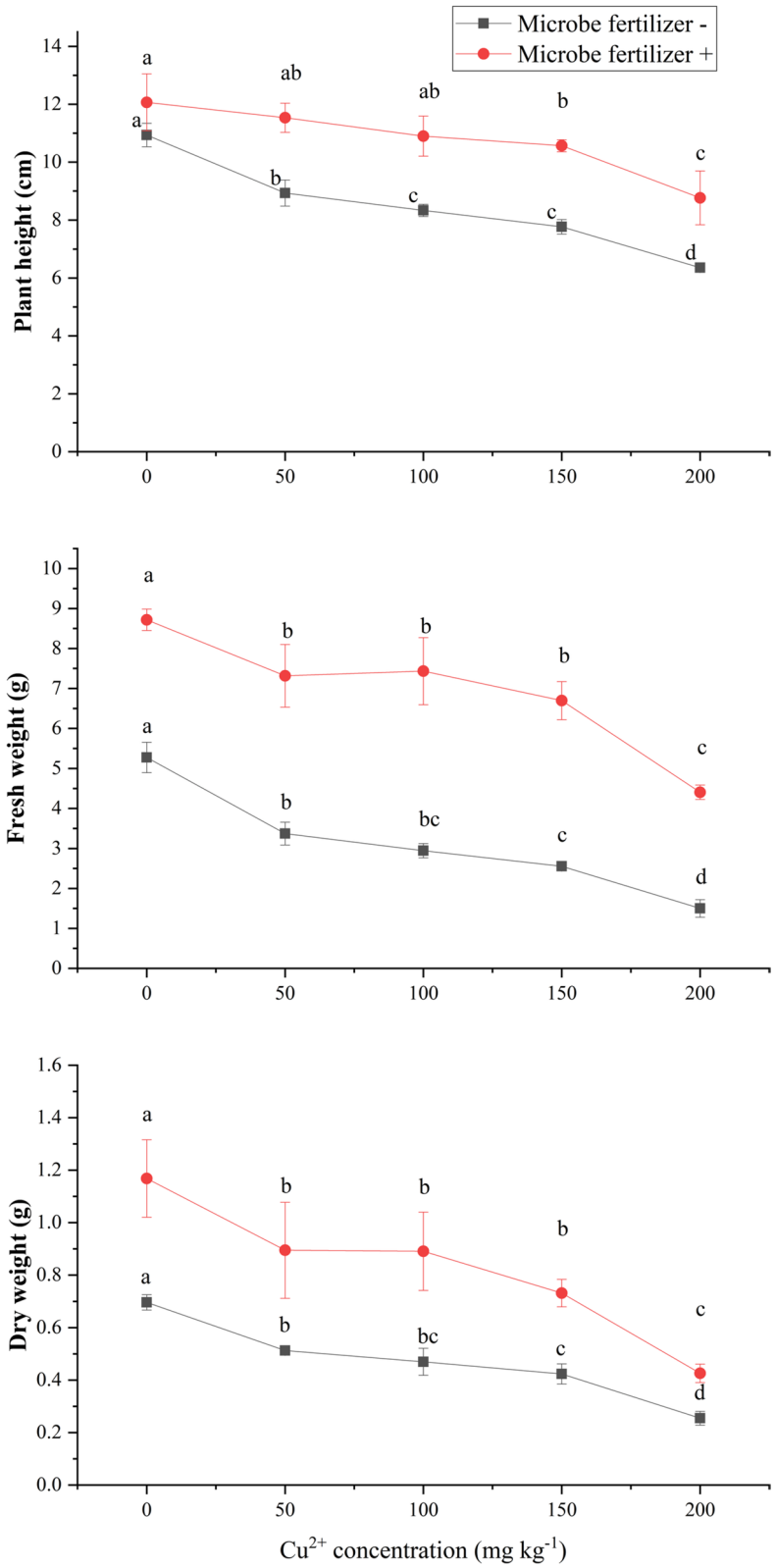

Fig. 5 Growth indicators of *B. chinensis* L. under biofertilizer treatment

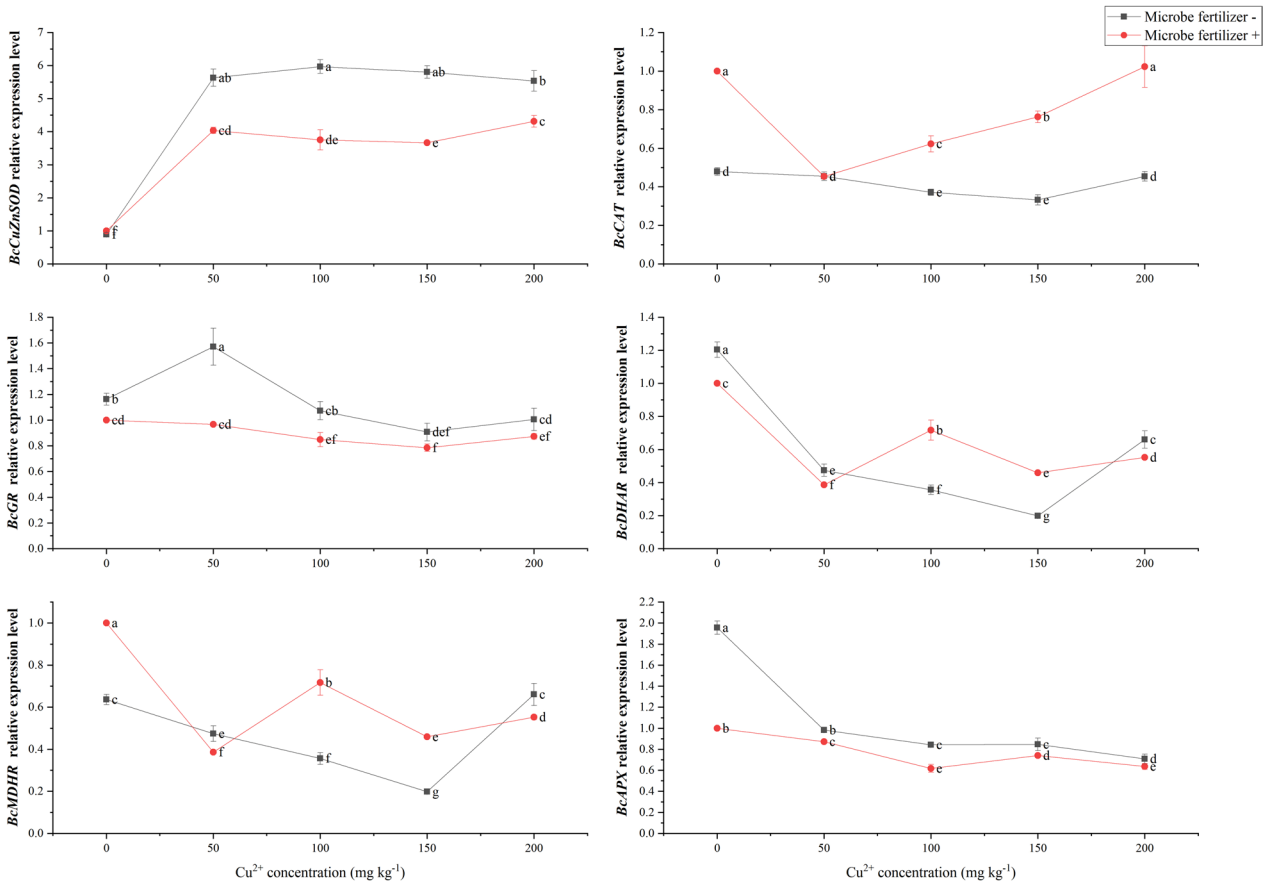

Fig. 6 Effect of applying microbial fertilizer on antioxidant enzyme gene expression levels in *B. chinensis* L

The microorganisms and organic matter contained in biofertilizers have a strong adsorption effect on metal ions, which can reduce the availability of heavy metals in soil and reduce the number of heavy metals absorbed by crops. Therefore, the use of microbial fertilizer is an effective measure for the passivation and remediation of heavy metal-contaminated soil. It is reported that maize and sunflower were treated with 10 mg L^{-1} Cd^{2+}, and compared with the control group, the application of biofertilizer could significantly reduce the absorption of Cd^{2+} by corn and sunflower (Gajdos et al. 2012). Adding biofertilizer decreased the content of soil extractable Cd^{2+} (Wang et al. 2019). We have also obtained similar results. Furthermore, when the copper concentration exceeds 100 mg kg^{-1}, it is inconsistent with previous research findings (Abbaszadeh-Dahaji et al. 2021). The likely reason for this is the use of different extractants. The strong acid HCl, which was used in this study, had a detrimental effect on soil pH, thereby facilitating the extraction of some Cu^{2+} ions that were fixed by high pH. Another possibility is that the high concentration of Cu^{2+} ions inhibits the normal growth and metabolism of *Bacillus amyloliquefaciens* SYNU1.

Copper (Cu) is an important essential micronutrient for plant growth and development. However, it can easily lead to poisoning of plants at high concentrations. A common consequence of Cu poisoning is the rapid and excessive accumulation of reactive oxygen species (ROS) due to interference with photosynthetic and respiratory electron transport activities, especially that of chloroplast membranes. High levels of ROS expose cells to oxidative stress, leading to cell membrane dismantling. It has been previously reported that plants resort to a complex defence mechanism to counteract oxidative stress caused by Cu exposure. For example, plants possess very efficient enzymatic antioxidant defence systems. The components of enzymatic antioxidants include SOD, CAT, APX, MDHAR, DHAR and GR, which work in concert to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging ROS (Gill and Tuteja 2010).

Superoxide dismutase (SOD) is the most effective intracellular enzymatic antioxidant and is ubiquitous in all aerobic organisms. SOD is the first line of defence against the toxic effects of elevated levels of ROS. SODs remove O_2^- by catalysing its dismutation, with one O_2^- being reduced to H_2O_2 and another oxidized to O_2 (Bowler et al. 1994). Catalases (CAT) are indispensable for ROS detoxification during stress conditions. It can directly dismutate H_2O_2 to H_2O and O_2. It has a high turnover rate, and one molecule of CAT can convert approximately 6 million molecules of H_2O_2 to H_2O and O_2 per minute (Willekens et al. 1997; Gill and Tuteja 2010). Ascorbate peroxidase (APX) is thought to play the most essential role in scavenging ROS and protecting cells in higher plants, which is involved in scavenging H_2O_2 in the water–water and ASH-GSH cycles and utilizes ASH as the electron donor (Asada 1992). Glutathione reductase (GR) is a flavoprotein oxidoreductase found in both prokaryotes and eukaryotes. It is a potential enzyme of the ASH-GSH cycle and plays an essential role in the defence system against ROS by sustaining the reduced status (GSH) (Romero-Puertas et al. 2006).

Conclusion

In summary, the effectiveness of biofertilizer with SYNU1 on the growth of Pakchoi planted in copper-contaminated soil was evaluated. Based on morphological, biochemical, physiological and phylogenetic analyses, SYNU1 was identified as *Bacillus amyloliquefaciens*. SYNU1 has a high adsorption capacity for copper, the maximum adsorption capacity of copper by strain SYNU1 is 53.09% w/w. The inoculation with the biofertilizer prepared by SYNU1 significantly alleviated the toxicity of copper on *B. chinensis* L. by decreasing *CuZnSOD* gene expression, and increased plant height, plant fresh and dry weight which were attributed to the increasing of *BcCAT1* gene expression. However, the available copper content of soil treated with biofertilizer was not significantly reduced compared with that of soil treated without biofertilizer. The reason for this is unclear and needs to be further studied.

Acknowledgements
Not applicable.

Authors' contributions
HH: methodology; writing-original draft; Investigation; Resources. SB: methodology; writing-original draft; investigation; formal analysis. YW: investigation; resources; project administration. DT: methodology; formal analysis; conceptualization; supervision; writing-review and editing.

Funding
This work was supported by the Doctoral Research Start-up Fund Project of Liaoning Province [grant number 2022-BS-227] and the Shenyang Science and Technology Mission Project [grant number 23-411-2-09].

Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 8 September 2023 Accepted: 27 December 2023
Published online: 13 January 2024

References

Abbaszadeh-Dahaji P, Atajan FA, Omidvari M, Tahan V, Kariman K (2021) Mitigation of copper stress in maize (Zea mays) and sunflower (Helianthus annuus) plants by copper-resistant Pseudomonas strains. Curr Microbiol 78:1335–1343. https://doi.org/10.1007/s00284-021-02408-w

Andreazza R, Pieniz S, Wolf L, Lee MK, Camargo FA, Okeke BC (2010) Characterization of copper bioreduction and biosorption by a highly copper resistant bacterium isolated from copper-contaminated vineyard soil. Sci Total Environ 408:1501–1507. https://doi.org/10.1016/j.scitotenv.2009.12.017

Asada K (1992) Ascorbate peroxidase- a hydrogen peroxide-scavenging enzyme in plants. Physiol Plant 85:235–241

Bhardwaj D, Ansari MW, Sahoo RK, Tuteja N (2014) Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. Microb Cell Fact 13:66. https://doi.org/10.1186/1475-2859-13-66

Bowler C, Camp WV, Montagu MV, Inzé D, Asada K (1994) Superoxide Dismutase in Plants. Crit Rev Plant Sci 13:199–218

Chang CH, Yang SS (2009) Thermo-tolerant phosphate-solubilizing microbes for multi-functional biofertilizer preparation. Bioresour Technol 100:1648–1658. https://doi.org/10.1016/j.biortech.2008.09.009

Choinska-Pulit A, Sobolczyk-Bednarek J, Laba W (2018) Optimization of copper, lead and cadmium biosorption onto newly isolated bacterium using a Box-Behnken design. Ecotoxicol Environ Saf 149:275–283. https://doi.org/10.1016/j.ecoenv.2017.12.008

Chun J, Bae KS (2000) Phylogenetic analysis of Bacillus subtilis and related taxa based on partial gyrA gene sequences. Antonie Van Leeuwenhoek 78:123–127. https://doi.org/10.1023/a:1026555830014

Derakhshan Nejad Z, Jung MC, Kim KH (2018) Remediation of soils contaminated with heavy metals with an emphasis on immobilization technology. Environ Geochem Health 40:927–953. https://doi.org/10.1007/s10653-017-9964-z

Fox GE, Wisotzkey JD, J., P.J., (1992) How close is close: 16s rRNA sequence identity may not be sufficient to guarantee species identity. Int J Syst Bacteriol 42:166–170

Gadd GM, Rome L (1988) Biosorption of copper by fungal melanin. Appl Microbiol Biotechnol 29:610–617

Gajdos É, Lévai L, Veres S, Kovács B (2012) Effects of biofertilizers on maize and sunflower seedlings under cadmium stress. Commun Soil Sci Plant Anal 43:272–279. https://doi.org/10.1080/00103624.2011.638591

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–930. https://doi.org/10.1016/j.plaphy.2010.08.016

Gong Q, Li Z, Wang L, Zhou J, Kang Q, Niu D (2021) Gibberellic acid application on biomass, oxidative stress response, and photosynthesis in *spinach* (*Spinacia oleracea L.*) seedlings under copper stress. Environ Sci Pollut Res Int 28:53594–53604

Han L, Li J, Xue Q, Chen Z, Zhou Y, Poon CS (2020) Bacterial-induced mineralization (BIM) for soil solidification and heavy metal stabilization: a critical review. Sci Total Environ 746:140967–140967

Haneef I, Faizan S, Perveen R, Kausar S (2014) Impact of bio-fertilizers and different levels of cadmium on the growth, biochemical contents and lipid peroxidation of Plantago ovata Forsk. Saudi J Biol Sci 21:305–310. https://doi.org/10.1016/j.sjbs.2013.12.005

Hansda A, Kumar V, Anshumali, (2015) Biosorption of copper by bacterial adsorbents: a review. Res J Environ Toxicol 9:45–58

Hossain MdS, Abdelrahman M, Tran CD, Nguyen KH, Chu HD, Watanabe Y, Hasanuzzaman M, Mohsin SM, Fujita M, Tran L-SP (2020) Insights into acetate-mediated copper homeostasis and antioxidant defense in *lentil* under excessive copper stress. Environ Pollut 258:113544

Kim BJ, Lee SH, Lyu MA, Kim SJ, Bai GH, Chae GT, Kim EC, Cha CY, Kook YH (1999) Identification of *mycobacterial* species by comparative sequence analysis of the RNA polymerase gene (rpoB). J Clin Microbiol 37:1714–1720

Leng X, Jia H, Sun X, Shangguan L, Mu Q, Wang B, Fang J (2015) Comparative transcriptome analysis of grapevine in response to copper stress. Sci Rep 5:17749. https://doi.org/10.1038/srep17749

Li D, Xu X, Yu H, Han X (2017) Characterization of Pb(2+) biosorption by psychrotrophic strain *Pseudomonas sp.* I3 isolated from permafrost soil of Mohe wetland in Northeast China. J Environ Manage 196:8–15. https://doi.org/10.1016/j.jenvman.2017.02.076

Wang L, Cheng WC, Xue ZF (2022) The effect of calcium source on Pb and Cu remediation using enzyme-induced carbonate precipitation. Front Bioeng Biotechnol 10:849631. https://doi.org/10.3389/fbioe.2022.849631

Liu YG, Liao T, He ZB, Li TT, Wang H, Hu XJ, Guo YM, He Y (2013) Biosorption of copper(II) from aqueous solution by *Bacillus subtilis* cells immobilized into chitosan beads. Trans Nonferrous Metals Soc China 23:1804–1814. https://doi.org/10.1016/S1003-6326(13)62664-3

Özdemir S, Kılınç E, Poli A, Nicolaus B (2013) Biosorption of Heavy Metals (Cd^{2+}, Cu^{2+}, Co^{2+}, and Mn^{2+}) by *Thermophilic* bacteria, Geobacillus thermantarcticus and Anoxybacillus amylolyticus: equilibrium and kinetic studies. Bioremediat J 17:86–96. https://doi.org/10.1080/10889868.2012.751961

Patsikka E, Kairavuo M, Sersen F, Aro EM, Tyystjarvi E (2002) Excess copper predisposes photosystem II to photoinhibition in vivo by outcompeting iron and causing decrease in leaf chlorophyll. Plant Physiol 129:1359–1367. https://doi.org/10.1104/pp.004788

Peng H, Xie W, Li D, Wu M, Zhang Y, Xu H, Ye J, Ye T, Xu L, Liang Y, Liu W (2019) Copper-resistant mechanism of Ochrobactrum MT180101 and its application in membrane bioreactor for treating electroplating wastewater. Ecotoxicol Environ Saf 168:17–26. https://doi.org/10.1016/j.ecoenv.2018.10.066

Pilon M, Abdel-Ghany SE, Cohu CM, Gogolin KA, Ye H (2006) Copper cofactor delivery in plant cells. Curr Opin Plant Biol 9:256–263. https://doi.org/10.1016/j.pbi.2006.03.007

Puig S, Andres-Colas N, Garcia-Molina A, Penarrubia L (2007) Copper and iron homeostasis in Arabidopsis: responses to metal deficiencies, interactions and biotechnological applications. Plant Cell Environ 30:271–290. https://doi.org/10.1111/j.1365-3040.2007.01642.x

Romero-Puertas MC, Corpas FJ, Sandalio LM, Leterrier M, Rodriguez-Serrano M, Del Rio LA, Palma JM (2006) Glutathione reductase from pea leaves: response to abiotic stress and characterization of the peroxisomal isozyme. New Phytol 170:43–52. https://doi.org/10.1111/j.1469-8137.2006.01643.x

Singh S, Moholkar VS, Goyal A (2013) Isolation, identification, and characterization of a cellulolytic *Bacillus amyloliquefaciens* strain SS35 from Rhinoceros Dung. ISRN Microbiol 2013:728134. https://doi.org/10.1155/2013/728134

Stern BR, Solioz M, Krewski D, Aggett P, Aw TC, Baker S, Crump K, Dourson M, Haber L, Hertzberg R, Keen C, Meek B, Rudenko L, Schoeny R, Slob W, Starr T (2007) Copper and human health: biochemistry, genetics, and strategies for modeling dose-response relationships. J Toxicol Environ Health B Crit Rev 10:157–222. https://doi.org/10.1080/10937400600755911

Sun Q, Lin S, Liu G, Li P (2023) Biochar derived from post-adsorbent for immobilizing Cu and Cd in sediment: the effect on heavy metal species and the microbial community composition. Toxics 11:666. https://doi.org/10.3390/toxics11080666

Tamura K, Stecher G, Kumar S (2021) MEGA11: molecular evolutionary genetics analysis version 11. Mol Biol Evol 38:3022–3027. https://doi.org/10.1093/molbev/msab120

Tanaka K, Fukuda M, Amaki Y, Sakaguchi T, Inai K, Ishihara A, Nakajima H (2017) Importance of prumycin produced by *Bacillus amyloliquefaciens* SD-32 in biocontrol against cucumber powdery mildew disease. Pest Manag Sci 73:2419–2428. https://doi.org/10.1002/ps.4630

Tang X, Jiang J, Huang Z, Wu H, Wang J, He L, Xiong F, Zhong R, Liu J, Han Z, Tang R, He L (2021) Sugarcane/peanut intercropping system improves the soil quality and increases the abundance of beneficial microbes. J Basic Microbiol 61:165–176. https://doi.org/10.1002/jobm.202000750

Tsai SH, Liu CP, Yang SS (2007) Microbial conversion of food wastes for biofertilizer production with *thermophilic lipolytic* microbes. Renewable Energy 32:904–915

Veglio F, Beolchini F (1997) Removal of metals by biosorption: a review. Hydrometallurgy 44:301–316

Verma A, Shalu S, A., Bishnoi, N.R., Gupta, A., (2013) Biosorption of Cu (II) using free and immobilized biomass of Penicillium citrinum. Ecol Eng 61:486–490. https://doi.org/10.1016/j.ecoleng.2013.10.008

Marques DM, da Silva AB, Mantovani JR, Magalhães PC, de Souza TC (2019) Root morphology and leaf gas exchange in *Peltophorum dubium* (Spreng.) Taub. (Caesalpinioideae) exposed to copper-induced toxicity. S Afr J Bot 121:186-192

Wang LF (2014) Physiological and molecular responses to drought stress in rubber tree (*Hevea brasiliensis* Muell. Arg.). Plant Physiol Biochem 83:243–249. https://doi.org/10.1016/j.plaphy.2014.08.012

Wang LT, Lee FL, Tai CJ, Kasai H (2007) Comparison of gyrB gene sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the Bacillus subtilis group. Int J Syst Evol Microbiol 57:1846–1850. https://doi.org/10.1099/ijs.0.64685-0

Wang M, Duan S, Zhou Z, Chen S (2019) Alleviation of cadmium toxicity to tobacco (*Nicotiana tabacum*) by biofertilizers involves the changes of soil aggregates and bacterial communities. Ecotoxicol Environ Saf 169:240–247. https://doi.org/10.1016/j.ecoenv.2018.10.112

Li W, Zhang Y, Achal V (2022) Mechanisms of cadmium retention on enzyme-induced carbonate precipitation (EICP) of Ca/Mg: Nucleation, chemisorption, and co-precipitation. J Environ Chem Eng 10:107507. https://doi.org/10.1016/j.jece.107507

Willekens H, Chamnongpol S, Davey M, Schraudner M, Langebartels C, Montagu MV, Inze D, Camp WV (1997) Catalase is a sink for H_2O_2 and is indispensable for stress defence in C3 plants. EMBO J 16:4806–4816

Yamamoto S, Harayama S (1995) PCR amplification and direct sequencing of gyrB genes with universal primers and their application to the detection and taxonomic analysis of Pseudomonas putida strains. Appl Environ Microbiol 61:1104–1109

Yin T, Lin H, Dong Y, Wei Z, Li B, Liu C, Chen X (2021) Inhibition of cadmium releasing from sulfide tailings into the environment by *carbonate-mineralized bacteria*. J Hazard Mater 419:126479. https://doi.org/10.1016/j.jhazmat.2021.126479

Yruela I (2005) Copper in plants. Plant Physiol 17:145–156

Yruela I (2009) Copper in plants: acquisition, transport and interactions. Funct Plant Biol 36:409–430. https://doi.org/10.1071/FP08288

Li W, Zhang Y, Achal V (2022) Mechanisms of cadmium retention on enzyme-induced carbonate precipitation (EICP) of Ca/Mg: Nucleation, chemisorption, and co-precipitation. Journal of Environmental Chemical Engineering 10:107507. https://doi.org/10.1016/j.jece.2022.107507

Publisher's Note