Ecotoxicology and Environmental Safety 94 (2013) 1–7

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Characterization of Cu-tolerant bacteria and definition of their role in promotion of growth, Cu accumulation and reduction of Cu toxicity in *Triticum aestivum* L

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ARTICLE INFO

Article history:
Received 19 December 2012
Received in revised form
8 April 2013
Accepted 11 April 2013
Available online 30 May 2013

Keywords:
Cu-tolerant bacteria
Metal accumulation
Plant growth
Antioxidant response

ABSTRACT

The effects of Cu-tolerant bacteria strain USTB-O on Cu accumulation, plant growth and reduction of Cu toxicity in wheat seedlings *Triticum aestivum* L. were investigated. The strain was identified as belonging to *Bacillus* species and showed a specific tolerance to Cu through binding the Cu ions to the cell walls to reduce their entry into the cells. The bacteria not only increased Cu accumulation in wheat seedlings, but also secreted indole-3-acetic acid (IAA) and therefore promoted plant growth. Moreover, the bacteria effectively improved the antioxidant defence system to alleviate the oxidative damage induced by Cu. The bacteria promoted superoxide dismutase (SOD) in both shoots and roots to reduce superoxide radicals. The bacteria stimulated all enzymes activities under Cu exposure conditions, peroxidase (POD) and catalase (CAT) in shoots and ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) in roots were major enzymes to eliminate H_2O_2 in wheat seedlings.

Capsule

The main tolerance mechanism of Cu-tolerant bacterial (USTB-O) against Cu could involve Cu adherence to the surface of the cell wall. The strain has positive effects on promotion of wheat seedlings' growth, increase of Cu accumulation and reduction of Cu toxicity.

1. Introduction

Concentration of Cu in soils is usually in the range of 10–30 mg/kg (Fernández-Calviño et al., 2008). However, soils in the vicinity of mining areas or around metal-processing industries may be heavily contaminated with Cu. Cu is an essential nutrient element for plant growth and development (Demirevska-Kepova et al., 2004); however an excess of Cu^{2+} can generate reactive oxygen species (ROS), causing lipid peroxidation (malondialdehyde (MDA)) in plants, which consequently increases membrane permeability (Andrade et al., 2010; Kafel et al., 2010). The excess of Cu in soil also could induce the decline in the photosynthetic function and root hair formation, which results in a biomass reduction (Cambroll et al., 2011; Kopittke et al., 2008; Mateos-Naranjo et al., 2008).

Under oxidative stress conditions, plants first perceive the stimulus, generate and transmit signals and accordingly induce biochemical changes that adjust the metabolism (Azcón et al., 2009), and then develop an antioxidant defence system to protect themselves from metal-induced oxidative injury (Demirevska-Kepova et al., 2004). Ability of plants to withstand metal-induced oxidative stress significantly depends on the induction of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) (Gratão et al., 2005; Guo et al., 2006). SOD is responsible for the dismutation of the superoxide radical to generate H_2O_2 and O_2. CAT, POD and APX can convert H_2O_2 to water and O_2, and DHAR reduces dehydroascorbate (DHAR) to ascorbate. Changes in these activities of antioxidant enzymes are an adaptive response to metal toxicity.

Studies have indicated that interaction between bacteria and plant roots can greatly enhance the ability of plants to take up metals (Dary et al., 2010; Glick, 2010; He et al., 2010, 2009). *Elsholtzia splendens* inoculated bacterial strain MS2 had significantly greater concentrations of Cu in shoots and roots than uninoculated plants (Chen et al., 2005). Furthermore, it can also increase the tolerance of metals to plants. The promotion of metal tolerance in plants by inoculation with microbes can occur by transformation of the metals into less toxic forms (Carlot et al., 2001), reduction of metal uptake by plants (Vivas et al., 2006) or reduction of the amount of detrimental stress ethylene induced by heavy metals without affecting their uptake (Rajkumar and Freitas, 2008). The copper-resistant bacteria *Bacillus* sp. BC21 and *Arthrobacter* sp. MT16 have been shown to promote plant growth with the production of indole-3-acetic acid

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0147-6513/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2013.04.005

(IAA) (Lin et al., 2010). Bacterial strains can also to some extent relieve growth inhibition of plants caused by excess exposure of heavy metals (Gao et al., 2010; Kumar et al., 2009; Ma et al., 2009; Naik et al., 2012). Nevertheless, it is still unclear whether bacteria are useful to detoxification of plant cells and whether they are useful for plants to resist heavy metal toxicity.

In the present work, a strain (USTB-0) was isolated from soil heavily contaminated with Cu and identified. Considering it is a Cu-tolerant strain isolated from the natural environment rather than a domesticated strain, the mechanism of Cu tolerance in the bacteria was investigated. Furthermore, to fully understand the role of the bacteria in detoxification of Cu in plants, effects of its inoculation on plant growth, Cu uptake and antioxidant response of wheat seedlings (*T. aestivum* L.) exposed to a series of concentrations of Cu were investigated in hydroponics experiments.

2. Materials and methods

2.1. Identification of Cu-tolerant bacteria

The Cu-tolerant bacteria were isolated from a Cu contaminated soil collected from the Tonglushan copper mine. The concentration of Cu in the soil was reported in the range of 1645–8950 mg kg^{-1} (Zheng and Kang, 2009). A 10-g portion of the soil sample was suspended in 90 mL sterile water and 0.1 mL serial dilutions (10^{-3}–10^{-5}) were spread on beef peptone (BP, pH 6.0) medium plates containing 10.0 g L^{-1} beef extract, 10.0 g L^{-1} peptone, 5.0 g L^{-1} sodium chloride, and 2 g L^{-1} agar. The plates were incubated at 35 °C for 2 days and morphologically different colonies appearing on the medium were isolated.

The selected strain was inoculated into the Cu BP medium containing $CuSO_4$ at the concentrations of 20, 40, 80, 160, 200, 240, 280, 320, 360, 400, 440, 560, 640 mg L^{-1}, and incubated at 35 °C for 2 days. The highest Cu-tolerant concentration of strain was measured.

The strain was inoculated in the series liquid BP to test the optimization of culture conditions (temperature, pH, NaCl). The value of OD 600 nm were recorded when the optimum temperatures were at 25, 30, 32, 35, 37, 40 °C (pH 6.0); the optimum pH were selected at pH 5.0, 6.0, 7.0, 8.0, 9.0 (35 °C); and NaCl concentrations (w/v) were used 0, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0% (35 °C, pH 6.0), respectively.

The strain was identified by 16S ribosomal DNA (16S rDNA) sequence analysis. For the 16S rDNA analysis, genomic DNA was extracted and 16S rDNA was amplified in polymerase chain reaction (PCR) using the genomic DNA as template and bacterial universal primers, (primer 1: 5′-AGAGTTTGATCCTGGCTCAG-3′; primer 2: 5′-TACCTT GTTACGACTT-3′). The amplification reaction was cycled at 94 °C for 1 min; 50 °C for 1 min and 72 °C for 1 min for 35 cycles. After amplification the reaction product was purified by using PCR purification kit (Shenggong Company, China) and sequencing was performed by Shengong Co., Ltd., Shanghai (China). The sequence was then compared to the similar sequences in the database using BLAST analysis (Basic local alignment and search tool, BLAST at NCBI). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.

2.2. Electron microscopy

Scanning electron microscopy with energy dispersive X-ray microanalysis (SEM/EDX) was used to examine the surface structure and metal distribution of the strain culture with 50 mg L^{-1} $CuSO_4$ BP media after 24 h. The analysis was performed by an FEI Quanta 400FEG high resolution scanning electron microscope (SEM) equipped with an energy dispersive spectroscopy X-ray microanalysis system y (energy dispersive spectrometer EDAX Genesis X4M). Analyses were performed using an accelerating voltage of 15 kV.

2.3. Accumulation of Cu ions in the selected Cu-tolerant strain

Bacteria were inoculated into the liquid BP for 14–16 h with shaking at 200 rmp. One mL bacteria culture was then inoculated in 100 mL Cu BP (pH 6.0) containing 50 mg L^{-1} $CuSO_4$ at 35 °C for 24 h. 100-mL fraction was collected at the intervals of 4, 6, 8, 11, 20 and 24 h, centrifuged at 10,000*g* for 5 min. Bacterial growth curves were then plotted the fresh weight of the harvested cells against time intervals. Following a 15-min desorption period, the harvest cells were suspended by ice-cold water or desorption Tris-Mes buffer solution (Tris-Mes 2 mM; $CaCl_2$ 0.5 mM, pH 7.2) according to the method of Nilesh et al. (2005) with minor modification. All the samples were then recentrifuged at 10,000*g* for 5 min and collected for Cu analysis.

2.4. Plant cultivation and exposure

Seeds of wheat (*T. aestivum* L.) were obtained from market. Prior to germination, the seeds were surface-sterilized with 70% (v/v) ethanol for 1 min and 2% (v/v) sodium hypochlorite for 10 min and rinsed thoroughly with sterile distilled water for at least five times. The seeds were germinated in the dark on moist filter paper at 22 °C for 2 days. And then, the seeds were transferred to a complete Hoagland nutrient solution (pH 6.0) for 12 days (Hoagland and Arnon, 1938). The Cu-tolerant bacteria were inoculated into the liquid BP for 12 h at 35 °C. The nutrient solution was renewed daily. Temperature in the growth chamber was 25/22 °C day/night and photon flux density at the plant level was 180 μ mol m^{-2} s^{-1} of photosynthetic active radiation (400–700 nm) during a photoperiod of 16 h.

Experiments were set up in triplicate and each replicate contained 10 uniform wheat seedlings. There were four sets of experiments, each having different culture condition: CK (BP only); Cu (BP and 50 mg L^{-1} $CuSO_4$); Cu-tolerant bactria (BP and Cu-tolerant bacteria) and Cu+Cu-tolerant bactria (BP with 50 mg L^{-1} $CuSO_4$ and Cu-tolerant bacteria). The 14 d-seedlings were cultured in the CK, Cu, Cu-tolerant bactria and Cu+Cu-tolerant bactria solutions for another 8 days, respectively. Five parallel samples were treated by above each solution. Every sample was contained 10 seedlings and 1 L above solutions in one plastic pot. Plants and culture solution from each sample were collected and analyzed at 0, 2, 4, 6 and 8 days, respectively. After washing with Tris–Mes buffer solution and sterile water, both plant shoot and root lengths were measured and recorded. The whole plants were then dried in an oven at 60 °C for 48 h and dry weights were recorded.

2.5. Cu analysis by ICP-MS

Bacterial and plant samples were digested in 3 mL HNO_3 and 1 mL $HClO_4$ and analyzed for Cu concentration using an ICP-MS (Plasma Quad III, Fisons Instruments, UK). A solution of ^{115}In at 10 ng mL^{-1} in 2% HNO_3 was used as an internal standard to compensate for matrix suppression and signal drifting during analysis.

2.6. Determination of indole acetic acid (IAA)

IAA was determined according to the method of Ma et al. (2009). After incubation, 1 mL culture solution was transferred into a tube and mixed vigorously with 2 mL of $FeCl_3$–$HClO_4$ reagent and incubated at 37 °C for 25 min, afterwards a pink color developed in the culture medium. The absorbance of pink color was read at 530 nm. IAA concentration in culture medium was determined using a calibration curve of pure IAA as a standard following the linear regression analysis.

2.7. Antioxidant response in wheat seedlings

Plant materials (1–2 g, shoots or roots) were homogenized in 50 mM potassium phosphate buffer (pH 7.0) at 4 °C. Homogenate was filtered through four layers of cheese cloth and centrifuged at 15,000 × g for 15 min at 4 °C. Supernatant was analyzed for lipid peroxidation, enzyme activity and protein.

Lipid peroxidation was determined by estimation of the malondialdehyde (MDA) content following the method of Mishra et al. (2006) with slight modification. The absorbance of the MDA was read at 532 nm and the nonspecific absorption at 600 nm was subtracted. The concentration of MDA was calculated at its extinction coefficient (155 mM^{-1} cm^{-1}).

Protein content of the supernatant was measured following the method of Demirevska-Kepova et al. (2004). The absorbance of the protein was read at 595 nm.

The activity of superoxide dismutase (SOD; EC 1.15.1.1) was assayed by the method of Beauchamp and Fridovich (1971). The change in absorbance was monitored at 560 nm and enzyme activity was expressed as μmol reduction of NBT min^{-1} g^{-1} fw. The peroxidase (POD; EC 1.11.1.7) activity was measured by the method of Srivastava and Souza (2010). Decrease in the absorbance was taken at 470 nm. Enzyme activity was expressed as μmol of H_2O_2 degraded min^{-1} g^{-1} fw. Catalase (CAT, EC 1.11.1.6) activity was measured according to Ding et al. (2008). The decrease in H_2O_2 was monitored through absorbance at 240 nm. Enzyme activity was expressed as μmol of H_2O_2 degraded min^{-1} g^{-1} fresh weight (fw). The activity of ascorbate peroxidase (APX; EC 1.11.1.11) was measured according to the method of Nakano and Asada (1981). The change in absorbance was monitored at 290 nm and enzyme activity was expressed as μmol of ascorbate oxidized min^{-1} g^{-1} fw. The activity of dehydroascorbate reductase (DHAR; EC 1.8.5.1) was measured according to the method of Srivastava and Souza (2010). The change in absorbance was monitored at 265 nm and enzyme activity was expressed as μmol of ascorbate deoxidized min^{-1} g^{-1} fw.

2.8. Statistical analysis

All experiments were repeated three times. Results are given as the mean values ± SD. Analysis of variance and the Student–Newman–Keuls test ($p < 0.05$) were used to compare treatment means. All the statistical analyses were carried out using SPSS 13.0.