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Increased growth and root Cu accumulation of *Sorghum sudanense* by endophytic *Enterobacter* sp. K3-2: Implications for *Sorghum sudanense* biomass production and phytostabilization

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ABSTRACT

Endophytic bacterial strain K3-2 was isolated from the roots of *Sorghum sudanense* (an bioenergy plant) grown in a Cu mine wasteland soils and characterized. Strain K3-2 was identified as *Enterobacter* sp. based on 16S rRNA gene sequence analysis. Strain K3-2 exhibited Cu resistance and produced 1-aminocyclopropane-1-carboxylate (ACC) deaminase, indole-3-acetic acid (IAA), siderophores, and arginine decarboxylase. Pot experiments showed that strain K3-2 significantly increased the dry weight and root Cu accumulation of *Sorghum sudanense* grown in the Cu mine wasteland soils. Furthermore, increase in total Cu uptake (ranging from 49% to 95%) of the bacterial inoculated-*Sorghum sudanense* was observed compared to the control. Notably, most of Cu (83–86%) was accumulated in the roots of *Sorghum sudanense*. Furthermore, inoculation with strain K3-2 was found to significantly increase Cu bioconcentration factors and the proportions of IAA- and siderophore-producing bacteria in the root interiors and rhizosphere soils of *Sorghum sudanense* compared with the control. Significant decrease in the available Cu content was also observed in the rhizosphere soils of the bacterial-inoculated *Sorghum sudanense*. The results suggest that the endophytic bacterial strain K3-2 may be exploited for promoting *Sorghum sudanense* biomass production and Cu phytostabilization in the Cu mining wasteland soils.

1. Introduction

Copper mine wastelands produced from copper ore processing have caused the release of heavy metals from the tailings into surrounding ecosystems and ground water (Andreazza et al., 2010; Meers et al., 2010). Copper mine wastelands are not suitable for the cultivation of food and feed crops and need remediation to reduce health risk to living organisms (Sheng et al., 2012). Ecological remediation of copper mine wastelands has received much attention around the world because it provides an ecologically sound and safe method for the restoration and remediation of copper tailings (Wong, 2003; Asensio et al., 2013; Babu et al., 2015). Furthermore, it may be very important to cultivate energy plants in the copper tailings for bioenergy plant biomass production and the restoration and remediation of copper mine wastelands.

Phytostabilization is an alternative cost-effective and the most realistic remediation strategy due to the immobilization of metals in soil through absorption and accumulation by the roots of plants (Santibáñez et al., 2008; Pérez-Esteban et al., 2014). The initial establishment of plants that can colonize mine tailings is important for successful phytostabilization or revegetation (Asensio et al., 2013). However, metal toxicity, low nutrient contents and poor physical structures can limit the vegetation establishment on mine tailings (Wong, 2003; Pérez-Esteban et al., 2014). This has prompted us to explore the possibilities of enhancing the metal resistance and accumulation of the metal-tolerant plants using plant growth-promoting bacteria (Sheng et al., 2012; Sun et al., 2010; Marques et al., 2013). Recently, the role of heavy metal resistant endophytic bacteria in the phytoremediation of heavy metal-contaminated soils has been reported (Sun et al., 2010; Zhang et al., 2011; Babu et al., 2013). Endophytic bacteria have the capacity to promote the plant growth and development under adverse conditions by using various mechanisms such as nitrogen fixation, production of IAA, siderophore, ACC deaminase, and arginine decarboxylase (Sun et al., 2010; Nassar et al., 2003; Arshad et al., 2007; Puente et al., 2009). Endophytic bacteria may be of particular interest as they have the advantage of being relatively protected from the competitive, high-stress environment of the

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soil (Babu et al., 2013; Sturz et al., 2000). Although bacterial-assisted phytoremediation has been studied (Dell'Amico et al., 2008; Mastretta et al., 2009; Chen et al., 2013), to date, no attempt has been made to screen Cu-resistant and plant growth-promoting endophytic bacteria from *Sorghum sudanense* (an energy plant with rapid growth, large biomass, high resistance to adverse conditions, and ease of cultivation) grown in the Cu mine wasteland and to evaluate the impact of the endophytic bacteria on the growth and Cu phytostabilization of *Sorghum sudanense* in the Cu mining wasteland. A better understanding of the characteristics of Cu-resistant and plant growth-promoting endophytic bacteria is needed for the development of efficient phytostabilization and energy plant biomass production of *Sorghum sudanense* in the Cu mine wastelands.

The objectives of this study were to isolate Cu-resistant and plant growth-promoting endophytic bacteria from the roots of *Sorghum sudanense* and to evaluate the impacts of the endophytic bacteria on the growth and Cu accumulation of *Sorghum sudanense* grown in a Cu mine wasteland soils.

2. Methods and methods

2.1. Isolation of Cu-resistant endophytic bacteria

Endophytic bacteria were isolated from surface-sterilized Cu tolerant *Sorghum sudanense* (*Sorghum bicolor* × *Sorghum sudanense*) plants grown in Cu mine wasteland soils. The isolation of Cu (10 mg L^{-1})-resistant endophytic bacteria from the roots of the plants was performed according to the method of Sun et al. (2010). Briefly, roots were sterilized by sequential immersion in 75% (v/v) ethanol and 1% mercuric chloride and washed with sterile water to remove surface sterilization agents. Roots (0.2 g) were ground by a mortar and pestle. Serial dilutions were spread on plates containing 1/5-strength LB medium with or without Cu. Plates were incubated for 7 days at 28 °C. Cu-resistant colonies were picked randomly and purified by streaking on the 1/5-strength LB media. The analyses of the Cu mine wasteland soils were as follows: soil pH was measured with a pH meter (PHS-3CT) after equilibrating 5 g of dry soils with 10 mL of deionized water for 30 min. Organic matter content, available N, P, K, water-soluble, NH_4OAc- and DTPA-extractable Cu contents were determined following the methods described in the Physical Chemical Analysis of soils (SSICA, 1980). Soil total Cu, Pb, and Zn were extracted with HF–$HClO_4$ (SSICA, 1980). The above Cu, Pb, and Zn concentrations in the extracts were determined with ICP-OES (inductively coupled plasma-optical emission spectrometer) (Optima 2100 DV; PerkinElmer, USA). Root and above-ground tissue Cu concentrations of *Sorghum sudanense* were determined according to the method of Sun et al. (2010).

2.2. ACC deaminase, IAA, siderophore, and arginine decarboxylase production

The ACC deaminase production of the Cu-resistant endophytic bacteria was evaluated according to the method of Glick et al. (1995). The production of IAA by the bacteria was determined according to the methods of Gordon and Weber (1951) and Sheng et al. (2008). The production of siderophores by the bacteria was determined according to the chrome azurol-S analytical method (Schwyn and Neilands, 1987; Manjanatha et al., 1992). Arginine decarboxylase production was detected in modified Moeller's decarboxylase agar medium supplemented with 1 g L^{-1} of L-arginine-monohydrochloride and 0.02 g L^{-1} phenol red as the pH dye indicator according the method of Sun et al. (2010).

Table 1
Plant growth-promoting characteristics of the endophytic bacterial strains.

Strain	IAA (mg L^{-1})	Siderophore[a]	ACC deaminase	Arginine decarboxylase	Cu MIC (mM)[b]
K1-6	54.9 ± 1.6	–	+	–	7.8 ± 0.5
K3-2	23.5 ± 2.1	+++	+	+	3.3 ± 0.2
K3-9	21.8 ± 0.7	++	+	+	1.6 ± 0.2

–, no detection.

[a] Siderophore production: + and ++, low; +++, high. Values of absorbancy/absorbancy reference at 630 nm: +, 0.8–1.0; ++, 0.6–0.8; +++, 0.4–0.6.

[b] MIC: minimal inhibitory concentration.

2.3. Minimum inhibitory concentration (MIC) of Cu of the bacteria

The MIC of Cu for the endophytic bacteria was determined by the plate dilution method as described by Summers and Silver (1972) and Aleem et al. (2003). Stock solution of $CuSO_4$ was prepared in double distilled water and sterilized. LB agar plates without Cu were used as controls. The lowest concentration that prevented bacterial growth was considered the MIC. The experiments were carried out in triplicate. Cultures were incubated at 28 °C for 7 days.

2.4. Identification of strain K3-2

K3-2 was finally selected as the most active strain for the experiments of plant growth and Cu accumulation of *Sorghum sudanense* based on the relative ability of the production of the plant growth-promoting factors and the Cu resistance of the endophytic bacteria (Table 1). The identification of strain K3-2 was made according to the method of Jiang et al. (2008). Briefly, DNA was extracted and 16S rDNA was amplified using the universal primers 27 f and 1492 r. The amplification products were purified and sequencing was performed. The 16S rDNA sequence was compared against the GenBank database using the NCBI Blast program (Altschul et al., 1997). The 16S rDNA sequence of strain K3-2 has been deposited in GenBank under accession number KJ631292.

2.5. Plant growth and Cu accumulation of Sorghum sudanense

Experiments were conducted in plastic pots filled with 6.0 kg of the Cu mine wasteland soils. Ten surface-sterilized seeds were placed in each pot at a 1.0 cm depth. After germination, plants were thinned to six plants per pot. For inoculation, strain K3-2 (Cu and chloroamphenicol resistance) was grown in liquid LB medium for 20 h at 28 °C, centrifuged, washed, and resuspended to 1×10^8 cells mL^{-1} in sterile distilled water. Bacterial suspension (15 mL pot^{-1}) was sprayed on the soil surface 10 d after seedling emergence. A dead bacterial (autoclaved at 121 °C for 30 min) inoculated sample was prepared as a control. Triplicate pots were used for each treatment. The pots were placed in a greenhouse. The average temperature of the greenhouse ranged from 20.3 °C to 31.4 °C, the relative humidity was 68.5%, and an average photoperiod was 10 h per day. The plants were harvested 60 days after inoculation. The rhizosphere and bulk soils were collected from each live or dead bacterial-inoculated treatment. The soil pH was determined according to the method of Sheng et al. (2012). Roots and aboveground tissues were separated and washed, first in several changes of 0.01 M EDTA and then in distilled water to remove any nonspecifically bound Cu and were divided into two portions. One portion of the roots and soils were used for the isolation of Cu-resistant bacteria according to the methods of Sun et al. (2010) and Jiang et al. (2008). Three hundred and sixty bacterial isolates were obtained (60 isolates were collected from each treatment, the number of collected isolates accounted for 91–