



Environmental Microbiology

Endophytic fungus *Purpureocillium* sp. A5 protect mangrove plant *Kandelia candel* under copper stress



Bin Gong*, Guixiang Liu, Rquan Liao, Jingjing Song, Hong Zhang

Qinzhou University, Guangxi Key Laboratory of Beibu Gulf Marine Biodiversity Conservation, Guangxi, China

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ABSTRACT

Mangrove is an important ecosystem in the world. Mangrove ecosystems have a large capacity in retaining heavy metals, and now they are usually considered as sinks for heavy metals. However, the mechanism of why the soil of mangrove ecosystems can retain heavy metal is not certain. In this research, endophytic fungus *Purpureocillium* sp. A5 was isolated and identified from the roots of *Kandelia candel*. When this fungus was added, it protected the growth of *K. candel* under Cu stress. This can be illustrated by analyzing chlorophyll A and B, RWC and WSD to leaves of *K. candel*. *Purpureocillium* sp. A5 reduces uptake of Cu in *K. candel* and changes the pH characterization of soil. Furthermore, A5 increase the concentration of Cu complexes in soil, and it enhanced the concentration of carbonate-bound Cu, Mn-Fe complexes Cu and organic-bound Cu in soil. Nevertheless, a significant reduction of the Cu ion was noted among A5-treated plants. This study is significant and illustrates a promising potential use for environmental remediation of endophytes, and also may partially explain the large capacity of mangrove ecosystems in retaining heavy metals.

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Introduction

Mangrove ecosystems are intertidal wetlands in sub-tropical or tropical temperate coastal zone. They possess a great commercial and ecological value for human. However, as a consequence of urban development, mangrove ecosystems have been subject to significant threat for contaminant input. Among the major pollutants from anthropogenic inputs are heavy metals.¹ Mangrove ecosystems have a large capacity in retaining heavy metals, and now they are often considered as sinks for heavy metals.

Elevated concentrations of copper have been recorded in mangrove sediments all over the world now.^{2,3} Uptake of excessive copper by mangrove plants may cause cellular damage, or lead to whole plant phytotoxic responses.¹ Furthermore, since copper cannot be degraded biologically, it is transferred and concentrated in tissues and pose long-term damaging effects on mangrove plants.

Endophytic fungi (or endophytes) are organisms that inhabit the healthy plants, and they have been isolated from almost all known plants.^{4–6} As for endophytes of mangrove plants, previous studies have concentrated on their diversity,^{4,7} occurrence and distribution,⁸ new metabolites,^{9,10}

* Corresponding author.

E-mail: 342965691@aliyun.com (B. Gong).

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and biological activity.^{11,12} Little information exists on their ecological effect on mangrove plants under heavy metals stress. In fact, many endophytes inhabit the roots of plants and their collaboration may play a key role in growing in the heavy metals contaminated environment. Hence, it is necessary to exploit the function of endophytes for the tolerance of mangrove plants to heavy metals.

Kandelia candel is a major mangrove species of the eastern group and are dominant along South China coast. The effects of endophytes on *K. candel* under copper stress condition have not been studied. In our work, the endophytes were isolated and identified from the copper stimulated root of *K. candel*, and the fungus was found to promote the growth of *K. candel* under Cu stress. To understand the mechanism of this phenomenon, pH, uptake of Cu in plant and the concentration of Cu complexes in soil were analyzed. Our research will be important for us to understand the ecological function of endophytes in mangrove ecosystems.

Materials and methods

Isolation and identification of endophytic fungi

The fungus used in this study was isolated from the roots of mangrove plants *K. candel* in Qinzhou city, China, according to the method described by Gong.¹³ The isolated endophytes were identified using monographs and molecular method described by Gong.¹⁴ Colonial morphology and macroscopic characteristics were recorded. The endophytes were identified through sequencing and phylogenetic analysis of Internal Transcribed Spacer (ITS). The DNA was extracted and purified using DNeasy Plant Mini Kit (Qiagen, Germany). The ITS sequence was amplified using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as described by White.¹⁵ The PCR products were purified using Microcon columns (OMEGA, USA), and sequenced by the service of Beijing San-boyuanzhi Company Ltd. (Beijing, China). The DNA sequence was checked for its homology by BLASTN program.¹⁶ The DNA sequence had been submitted to the GenBank under the accession number KT239373. To construct the relevant phylogenetic tree, MEGA 5.0 software was employed in this study. The alignment data was subsequently analyzed by the neighbor-joining (NJ) method (Kimura two-parameter distance calculation). The bootstrap was 1000 replications to assess the reliable level to the nodes of the tree.

Plant growth with and without endophytic fungi under copper stress

Propagule of *K. candel* was obtained from Qinzhou Harbor in Guangxi Province of China. Single hypocotyl was planted in free-metal plastic pots with sand washed with sterilized water and placed in a greenhouse in Qinzhou University, China. These plants were kept at a temperature of $24 \pm 2^\circ\text{C}$ and light intensities of $480 \mu\text{mol m}^{-2}$ from natural sunlight. The plots were fertilized with 0.6L 50% (v/v) Hoagland's solution with 50% sea water every 7 d. After three leaves had been came out. The seedlings were divided into ten groups (3 in each group).

Five groups of *K. candel* were irrigated with liquid fertilizer (as described above) containing 1 g filtered fungi mycelia and Cu at five levels, 0, 25, 100, 200 and 400 mg kg^{-1} , respectively; other five groups were irrigated with liquid fertilizer and Cu at five levels, 0, 25, 100, 200 and 400 mg kg^{-1} , without addition of A5. Each pot was irrigated with 0.5l of corresponding liquid once a week. After one month, leaves and roots were collected for analysis. Fungi were prepared as follows: endophyte was grown in 500 mL Erlenmeyer flasks containing 100 mL liquid broth (pH 6.2) for a period of 8 days at $27 \pm 2^\circ\text{C}$ at 200 rpm on an incubator shaker. The samples were harvested 8 days after inoculation. Mycelia and broth were separated by filtration.

Detection of chlorophyll A and B of *K. candel*

The concentrations of chlorophyll A and B of leaves were assayed according to Zhang.¹⁷ The chlorophyll was extracted from 200 mg fresh sample in 20 mL ethanol, acetone and water (4.5:4.5:1, v/v/v) mixture over night and measured at 645 nm and 663 nm with a UV spectrophotometer. The experiments were conducted in triplicates and were repeated three times.

Detection of RWC and WSD to leaves of *K. candel*

Relative water content (RWC) and water saturation deficit (WSD) of the leaves were estimated according to the method described by Zhu.¹⁸ RWC and WSD were calculated according to the following equations:

$$\text{RWC (\%)} = \frac{\text{FW} - \text{DW}}{\text{SW} - \text{DW}} \times 100;$$

$$\text{WSD (\%)} = 1 - \text{RWC (\%)}$$

FW stood for fresh weight, DW for dry weight, SW for saturated weight, and LW for loss weight. SW was determined after soaking in distilled water for one day and LW was measured after dehydrating for 24 h at the room temperature.

Determination of Cu concentration underground and up ground part of the plant

Plant samples were gathered according to the standard method as described by Van Loon.¹⁹ Oven-dried plant samples were sieved through muslin cloth. Ash was prepared at 400°C in a muffled furnace for 8 h. One gram cooled ashed sample was treated with 2 mL of 65% HNO_3 added 6 mL of HCl. After digestion on a sizzling plate, dense fume evolved and a clear solution was obtained. The clear solution was filtered through Whatman No. 42 filter papers. The filtrate was used for determination of the concentration of Cu by iCE 3000 atomic absorption spectrophotometer (Thermo Scientific, USA).

pH values and particulate Cu in Soil

The pH values of the soils were measured following the national standards.²⁰ Sediment samples were collected and used for further analysis immediately. Particulate Cu in Soil samples were prepared according to the method described by Tessier with slight modification.²¹ An analytical procedure

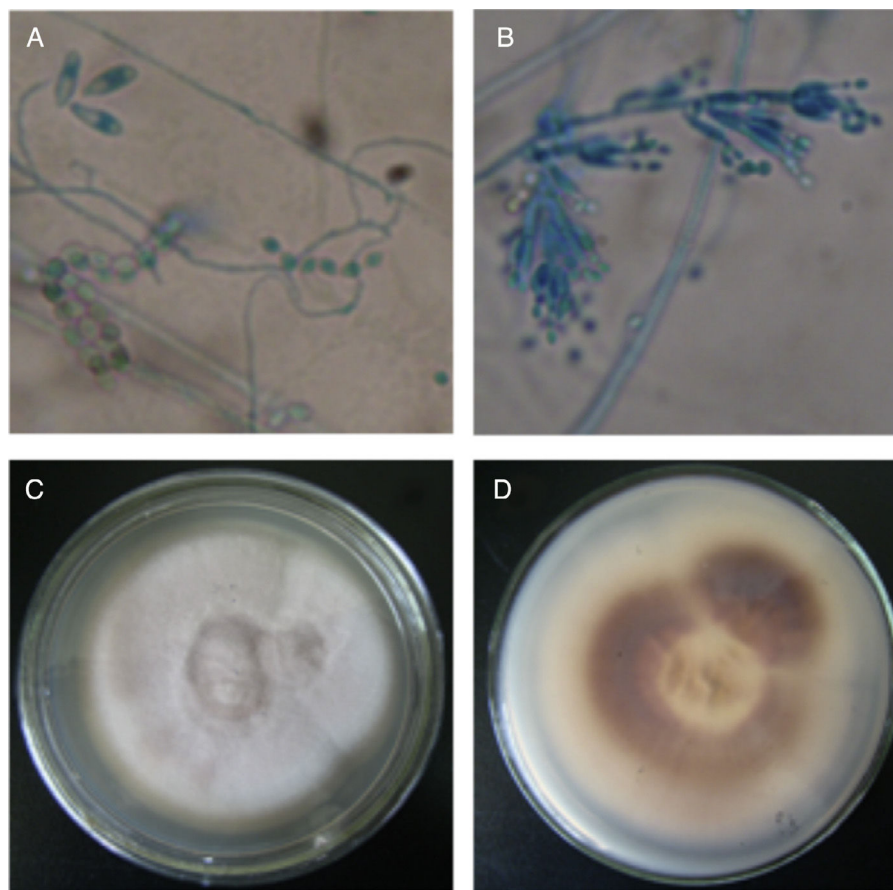


Fig. 1 – *Purpureocillium* sp. A5. (A) Short conidiophores of *Purpureocillium* sp. and those of the *Acremonium*-like synanamorph borne from the same hypha; (B) long conidiophores; (C and D) colony surfaces and reverse on PDA after 14 d incubation at 28 °C.

involving sequential chemical extractions had been applied for the partitioning of particulate Cu into four fractions: bound to Fe–Mn oxides, bound to organic matter, exchangeable, bound to carbonates. Chemical extraction methods were retained for further study, the quantities indicated below refer to 2-g sediment samples (dry weight of the original sample used for the initial extraction).

Statistical analysis

All data was tested and analyzed using SAS version 8.1 software package (SAS Institute Inc., USA). Comparisons between means were carried out using Duncan's multiple range tests at a significance level of $p < 0.05$. Graphical work was carried out using Sigma Plot v. 10.0.

Result and discussion

Isolation and identification of endophytic fungus *Purpureocillium* sp. A5 from the roots of *K. candel*

The endophytic fungus A5 was isolated from the roots of *K. candel* in Qinzhou City, China. Colonies of A5 on PDA consist of either basal or compact crustose felt in numerous

conidiophores with a floccose overgrowth of aerial mycelium. Colonies at first white, becoming pink and lilac with the onset of sporulation. Reverse in shades of purple. Conidial structures were shaped like typical *Purpureocillium lilacinum* phialides, or very long (up to 30 mm) and *Acremonium*-like. Conidiophores arising from superficial mycelium, monone-matous, stiff, verticillate; phialides ovate to cylindrical with erect and densely grouped. The morphological characteristics of A5 were consistent with *Purpureocillium* genus (Fig. 1).

The alignment of ITS sequence of A5 showed 100% homology with *P. lilacinum*. 14 available ITS sequences of the *Purpureocillium* genus were used for phylogenetic analysis. Fig. 2 shows the neighbor-joining analysis of the ITS sequences and two clades were present in this phylogram. The results demonstrate that *Purpureocillium* sp. MCM HumanF8-1 (AB915809) was phylogenetically closely related to *Purpureocillium* sp. A5. Based on morphology and ITS sequence analysis, A5 was identified as *Purpureocillium* sp. *Purpureocillium* is a well studied genus that commonly isolated from insects, soil, decaying vegetation, and cause infection in man and other vertebrates.²² Studies of the *Purpureocillium* as endophyte have not already been reported in previous literatures. In the present study, *Purpureocillium* sp. A5 can be considered to be true endophytes for the following two reasons, firstly

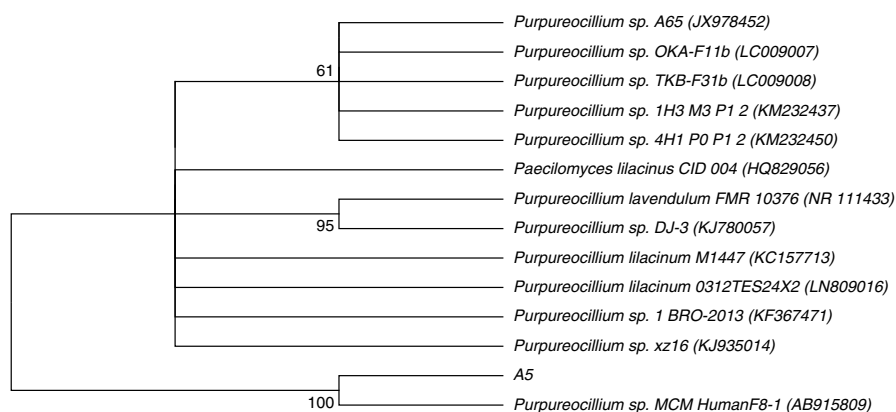


Fig. 2 – Neighbor-joining tree of the ITS sequences. The tree was constructed based on rDNA sequence (ITS1, 5.8S and ITS 4) by using the neighbor-joining method. Numbers above the nodes indicate bootstrap values (>50%) from 1000 replicates.

all surface-adhering microorganisms were removed by surface sterilization protocol, and secondly A5 was isolated from the roots of copper stimulated *K. candel*.

Purpureocillium sp. A5 promoted the growth of *K. candel* under Cu stress

Fig. 3 illustrates the growth condition of *K. candel* under different combinations of Cu and A5 treatment. A different growth response to Cu occurred among A5-treated and non-treated plants. When A5 and Cu were added, morphological characteristics were not significantly different among treatment from 0 to 400 mg kg⁻¹. However, without A5 treatment, the leaves wither and fall off the trees as Cu levels increased, and non-significant influence was observed at either 0 or 25 mg kg⁻¹.

The apparent difference was due to the chlorophyll, Relative Water Content (RWC) and Water Saturation Deficit (WSD) in leaves (Fig. 4). The addition of 0 and 100 mg kg⁻¹ Cu had negligible impact on chlorophyll A and B, while 25 mg kg⁻¹ slightly increased chlorophyll. In contrast, addition of A5 on 200 and 400 mg kg⁻¹ Cu treated plant resulted in strikes out of chlorophyll concentration. In general, RWC decreased as Cu concentration increased, but for A5 treated plants, RWC

declined by about 1.7- and 3.3-fold in 100 and 200 mg kg⁻¹ Cu treated plant. The addition of A5 also had apparent impact on WSD. With the addition of 25, 100 and 200 mg kg⁻¹ Cu, WSD was enhanced by about 6.5- and 4.8- and 3.1-fold, respectively.

Some fungi, such as Arbuscular Mycorrhizal in the root of plants, also have been found to promote the growth of plants in Cu contaminated environment.²³ The beneficial impacts of mycorrhizae colonization on plant growth could be largely explained by both improved P nutrition and decreased shoot Cu concentrations.²³ However, little is known about the endophytic fungi in root of plants and their growth promotion effect for planting under Cu stress.

Purpureocillium sp. A5 reduced uptake of Cu in *K. candel*

Cu contents in plants increased as the addition of Cu to the soil. However, differential up ground and underground Cu content occurred among A5-treated and non-treated plants (Fig. 5). The Cu concentration in up ground plant was not apparently affected at either 0, or 25 mg kg⁻¹ Cu, and then there was a slightly higher Cu content in non-treated plants. At 200 mg kg⁻¹ Cu, Cu content in the up ground parts of treated

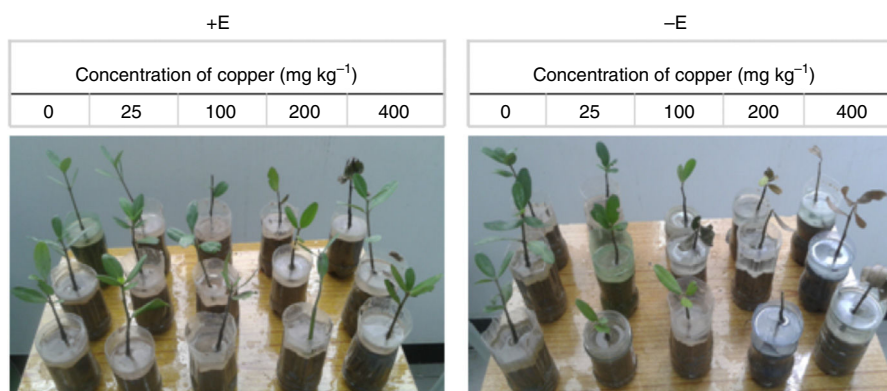


Fig. 3 – The effect of A5 on growth of *Kandelia candel* in copper stress. +E indicates A5 co-cultivated with *Kandelia candel* seedlings for 20 days in different concentration of copper stress. –E indicates *Kandelia candel* cultivated in the same condition with +E except for adding A5.

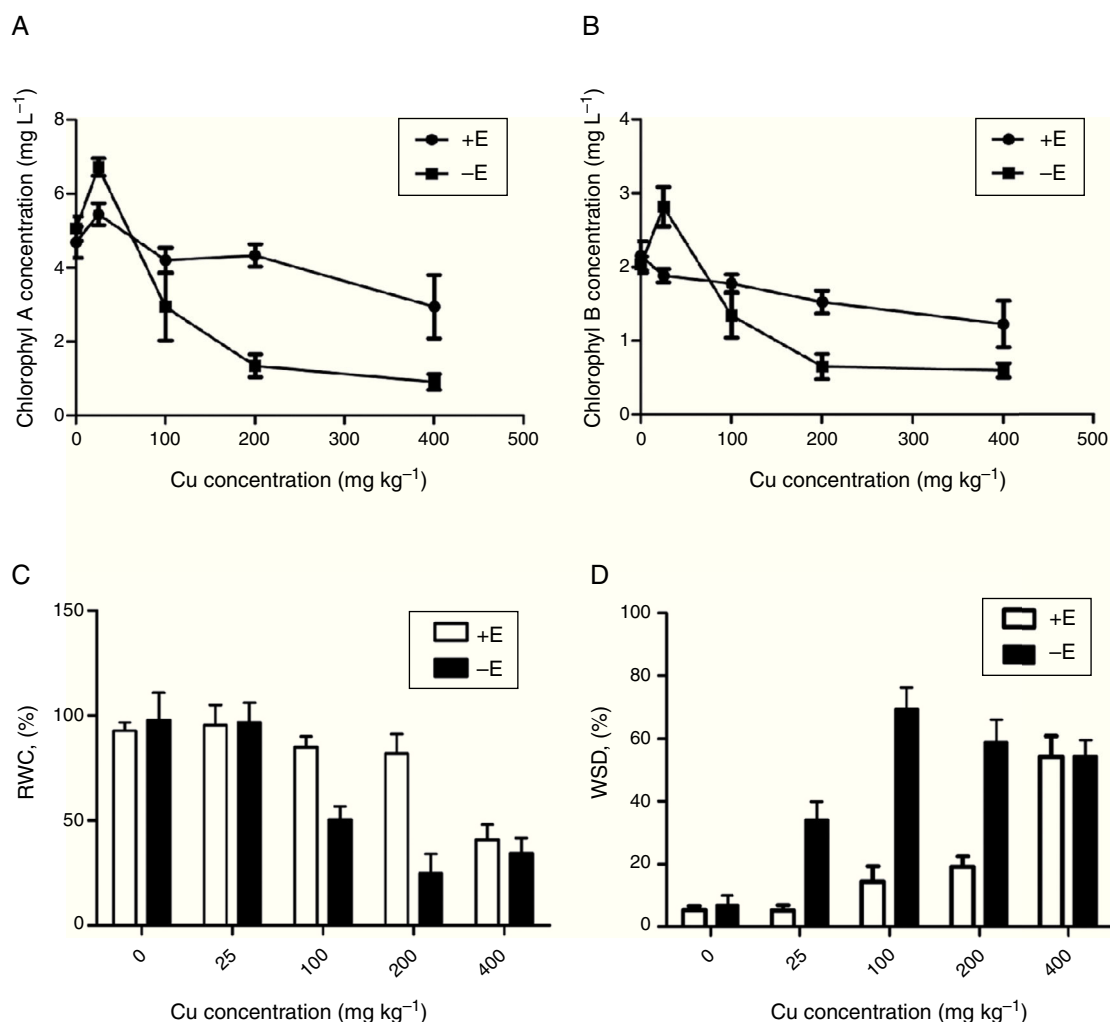


Fig. 4 – The effect of A5 on chlorophyll concentration, RWC and WSD in leaves of *Kandelia candel* in copper stress. (A) Chlorophyll A concentration under 0, 25, 100, 200, 400 mg kg⁻¹ of Cu with and without A5. (B) The chlorophyll B concentration under 0, 25, 100, 200, 400 mg kg⁻¹ of Cu with and without A5. (C) The effect of A5 on RWC to leaves of *Kandelia candel*. (D) The effect of A5 on WSD to leaves of *Kandelia candel*.

plants was 3.3-fold lower than non-treated plants. Cu concentrations in the roots rose significantly with copper addition from 0 to 200 mg kg⁻¹ in soil. When 0 and 25 mg kg⁻¹ Cu added, the underground Cu of A5-treated plants was nearly the same as control plants, but slightly higher in 100 mg kg⁻¹ Cu stress. At 200 mg kg⁻¹, Cu content in the underground parts of A5-treated plants was 2.3-fold lower than non-treated plants. The *Purpureocillium* sp. A5 substantially reduced absorption and uptake of Cu in *K. candel* under different copper stress.

Purpureocillium sp. A5 changed the pH characterization of soil

There was a significant influence of A5 on the pH value of soil (Fig. 6). When lower Cu concentration (0, 25 and 100 mg kg⁻¹ Cu) was supplied, no differences were found in pH of soil among A5-treated and non-treated groups. Nevertheless, at 200 mg kg⁻¹ Cu, in the absence of A5, pH in

soil was 1.42-fold higher than A5-treated plants. By addition of 400 mg kg⁻¹ Cu, the pH was not significantly different between A5-treated and non-treated plants, but there was a slightly lower pH than lower Cu treated groups. The addition of *Purpureocillium* sp. A5 changes the pH characterization of soil.

Purpureocillium sp. A5 increased the concentration of complexes Cu in soil

In order to illustrate the mechanism why A5 reduced the toxicity of copper to *K. candel*, the formation of the copper complexes in soil in 200 mg kg⁻¹ Cu stresses was studied (Fig. 7). In general, the Cu complexes increased as the A5 added. A5 treatment enhanced the concentration of carbonate-bound Cu, Mn-Fe Cu complexes and organic-bound Cu in soil by about 3.9-, 2.7- and 3.1-fold, respectively. Nevertheless, a significant reduction of the Cu ion was noted among A5-treated and non-treated plants by 4.8-fold. Maybe this fungus could secrete

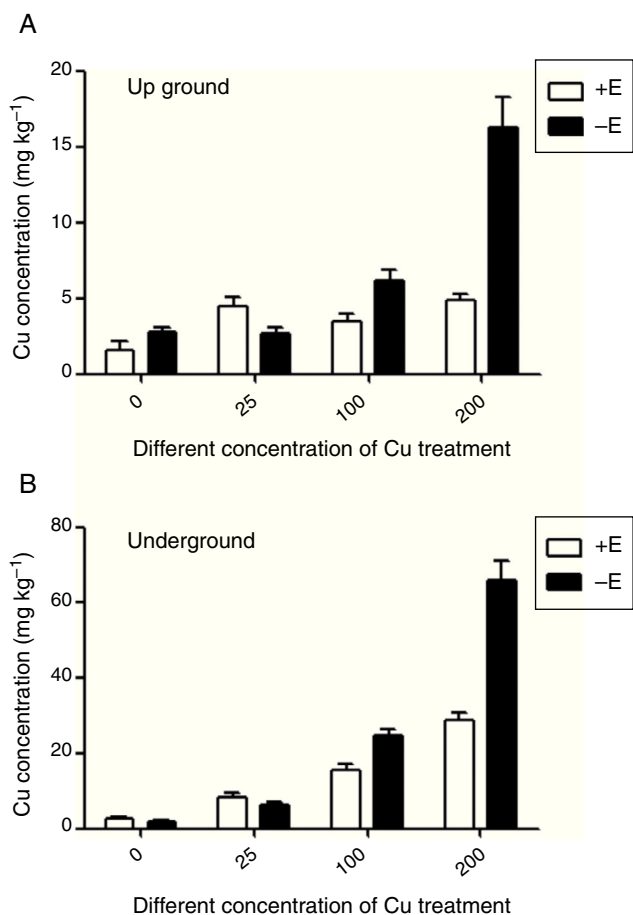


Fig. 5 – The effect of *Purpureocillium* sp. A5 on uptake of Cu in *Kandelia candel* in different copper stress. (A) The Cu concentration in up ground parts. (B) The Cu concentration in underground parts.

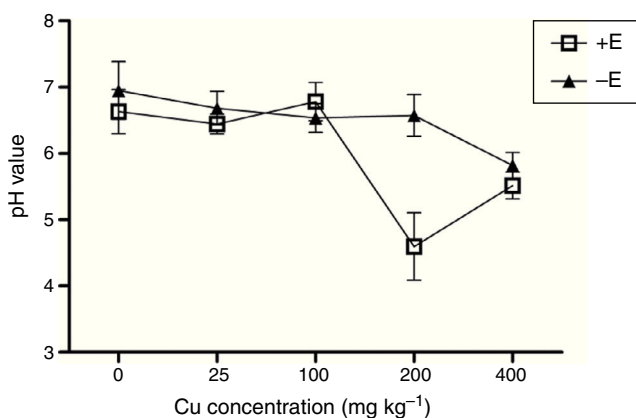


Fig. 6 – The effect of *Purpureocillium* sp. A5 on soil pH in different copper stress.

some acidic compound, because the addition of *Purpureocillium* sp. A5 decreased the pH value of soil. Furthermore, the acidic compound enhanced the formation of carbonate-bound Cu, Mn-Fe complexes Cu and organic-bound Cu, and significantly reduced the toxic Cu ion in soil.

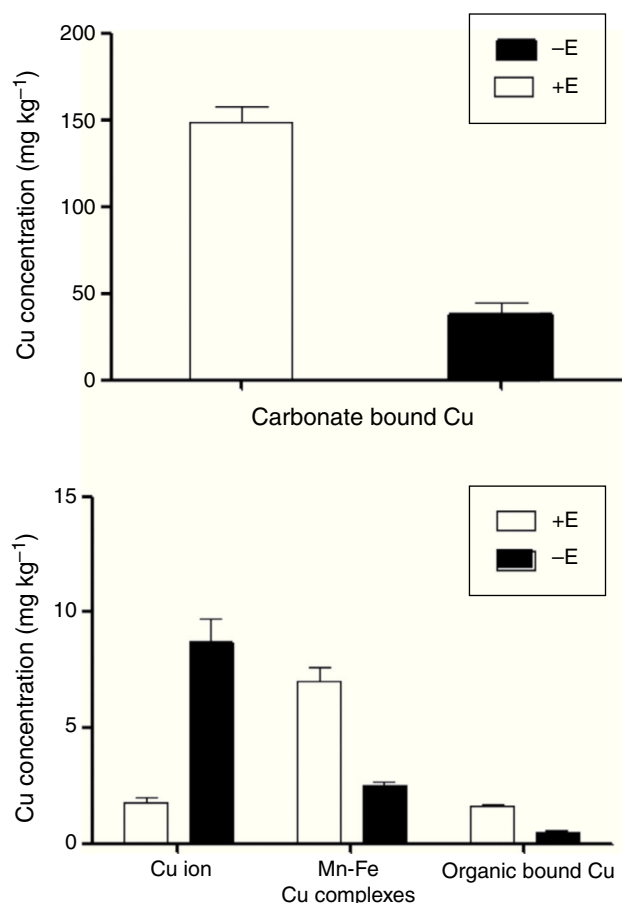


Fig. 7 – The effect of *Purpureocillium* sp. A5 on the formation of the copper complexes in 200 mg kg⁻¹ Cu stresses.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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