Root endophytic *Chaetomium cupreum* promotes plant growth and detoxifies aluminum in *Miscanthus sinensis* Andersson growing at the acidic mine site

Running title: Enhancement of Al tolerance by root endophyte

Toshikatsu Haruma,* Keiko Yamaji,* Hayato Masuya† and Kazuyo Hanyu*

**Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1, Tennoudai, Tsukuba 305-8572, Japan, and †Tohoku Research Center, Forestry and Forest Products Research Institute, Nabeyashiki, 92-25, Shimo-kuriyagawa, Morioka 020-0123, Japan*

Correspondence: Keiko Yamaji

Email: yamaji.keiko.fp@u.tsukuba.ac.jp

Facsimile and Telephone numbers: +81-29-853-7202

Member number: 1285

Abstract

Miscanthus sinensis Andersson grows naturally at the Hitachi mine. The root-zone soil was acidic and contained high concentrations of Cu, Pb, Zn and exchangeable Al. Adventitious roots accumulated high concentrations of Al and Fe, but not other heavy metals. The purpose of this study was to elucidate the mechanism of tolerance to Al in *M. sinensis*, focusing on its chemical interaction with root endophytes. We isolated *Chaetomium cupreum*, which produced siderophores, from adventitious roots of *M. sinensis* via CAS-assay. In inoculation tests, *C. cupreum* promoted *M. sinensis* seedling growth and increased Al and Fe uptake in the roots, although *C. cupreum* did not stimulate *M. sinensis* to produce Al detoxicants, such as citric and malic acids. Observation of the pattern of Al localization in the roots clarified that *C. cupreum* reduced Al toxicity in *M. sinensis* via compartmentalizing Al into fungal mycelia surrounding the roots and creating a less-toxic Al-localization pattern, allocating Al in the epidermis, endodermis, and stele of roots. In conclusion, our results indicated that *C. cupreum* increases Al tolerance in *M. sinensis* growing at the acidic mine site.

Key words: *Miscanthus sinensis* Andersson, *Chaetomium cupreum*, root endophyte, aluminum, siderophore.

Introduction

Since the late 1970s, acidification of ecosystems, including lakes, rivers, and forests, has become a concern globally because of acid rain caused by air pollutants, such as SO_x and NO_x, which have been emitted from industrial plants and smelters (Schindler 1988). Soil acidification inhibits plant growth, because Al, the most abundant metal in the earth's crust, is released from soil minerals into the soil solution as the toxic Al^{3+} (Kinraide 1991), which adversely affects plants (Delhaize & Ryan 1995; Kochian *et al*. 2005). Al uptake into plant cells is detrimental to plant growth, because of the inhibition of root elongation (Ryan *et al*. 1993) and reduction of the uptake of an essential nutrient, Ca (Delhaize & Ryan 1995). In our study site, the Hitachi mine, Ibaraki Prefecture, Japan, sulfur gas emitted during copper smelting caused severe damage in the 1900s, such as denuding the land and acidifying the soil (Japan Energy Co., Ltd. 1994). Currently, forest vegetation has been recovered by tree planting; however, the soil is still acidic and contains high concentrations of heavy metals, such as Cu, Pb, and Zn (Kubota *et al*. 1986), which also affect plant growth (Påhlsson 1989). Therefore, plants growing at the Hitachi mine seem to tolerate Al and heavy metal toxicities in the acidic soil.

Miscanthus sinensis, a common perennial plant in Japan, is easily observed at the Hitachi mine. *Miscanthus sinensis* is a pioneer species in disturbed ecosystems, such as volcanic areas with acid-sulfate soils, which are naturally caused by volcanic gases and volcanic products (An *et al*. 2008). Because of this, it was believed that *M. sinensis* would be an Al-tolerant species in acidic soils (Horie & Nemoto 1990). *Miscanthus sinensis* can accumulate high concentrations of Al without any symptoms, and an acidic water culture experiment showed that Al does not cause root growth inhibition in *M.*

sinensis, suggesting that this plant has Al tolerance mechanisms (Horie & Nemoto 1990). However, the mechanisms inside of plants are currently unknown. Under acidic soil conditions, heavy metals are also in bioavailable forms (Larcher 2003) and plants can absorb heavy metals more easily. *Miscanthus sinensis* is known to grow naturally at several mine sites in Japan (Hiroi 1974), therefore, *M. sinensis* growing at the Hitachi mine appears to have heavy metal tolerance as well.

Nonclavicipitaceous endophytes such as *Fusarium*, *Cryptosporiopsis*, and *Colletotrichum*, broadly and extensively colonizes shoots, roots, and rhizomes, and some of nonclavicipitaceous endophytes confer fitness benefits, such as drought, heat, and pathogen tolerance in plants (Rodriguez *et al*. 2009). Other type of endophytic fungi, dark septate endophytes, have distinctively dark color hyphae with septate (Jumpponen & Trappe 1998). They are reported to colonize approximately 600 plant species (Jumpponen & Trappe 1998), and improve heavy-metal tolerance in plants such as maize (Li *et al*. 2011a). Moreover, some root endophytes produce siderophores, which are reported to chelate various metals, and thus detoxify Al (Rogers *et al*. 2001) and other harmful metals (Rajkumar *et al*. 2012). Although Straub *et al*. (2013) reported that a bacterial endophyte promoted *M. sinensis* growth, there is no report of Al detoxification in *M. sinensis* interacting with fungal root endophytes. Therefore, in this study, focusing on chemical interaction between plants and root endophytes, we consider that root endophytes could protect *M. sinensis* from metal toxicities by the production of siderophores.

The objective of our study was to elucidate the mechanisms for Al tolerance in *M. sinensis* by investigating its chemical interactions with root endophytes at a study site, the Hitachi mine. First, we verified that *M. sinensis* accumulated high

concentrations of Al and much lower concentrations of Cu, Pb, and Zn. Therefore, we considered that *M. sinensis* by itself or through an interaction with root endophytes would have Al tolerance mechanisms. We isolated endophytes from the adventitious roots and examined siderophore production activities of isolates via CAS-assay. Among all isolates, *Chaetomium cupreum* produced large quantities of siderophores. We conducted two inoculation tests (soil inoculation test and water culture inoculation test) using *C. cupreum* and *M. sinensis* seedlings to clarify the effect of *C. cupreum* on Al tolerance mechanisms in *M. sinensis* via chemical and histochemical analyses. Additionally, we also discussed reasons why *M. sinensis* could survive in the acidic mine site, in terms of Al tolerance mechanisms.

Materials and methods

Study site, sampling, and analysis of elements in root-zone soil and plant tissues

The study site (36°37′N, 140°38′E, 100 × 58 m, southern slope) was located at the Hitachi mine, Ibaraki Prefecture, Japan. The soil was classified as a Cambisol according to the FAO-UNESCO system (FAO 2014). *Miscanthus sinensis* was the dominant species at our study site, and its herbaceous cover rate was 0.45 ± 0.04 m^2 [mean ± standard error (SE), 116 plots (1 × 1 m each)]. In July 2012, we arbitrarily set five sampling points (5 × 5 m each).

In August 2012, the root-zone soil (200 × 200 × 200 mm volume, the observed area of the root system including adventitious roots of *M. sinensis*) excluding litter was collected from five sampling points as described above; monthly-analysis of heavy

106 metal concentrations in the root-zone soil would not be needed because there was not
107 significant seasonal variation shown as the previous researches (Nagata *et al*. 2015;
108 Yamaji *et al*. 2016a). Ecological correspondence for soil and plants should be
109 considered (Verheyen *et al*. 2003), thus the root-zone soil simultaneously collected with
110 plants in August 2012 were used for analysis of the soil properties. After being air-dried
111 at 20°C for 1 week, the soil was passed through a sieve (<2 mm). We measured soil
112 properties: pH (H_2O), concentrations of total Al and heavy metals (Cd, Cu, Fe, Ni, Pb,
113 and Zn), cation exchange capacity (CEC), organic C, and total N. Using a pH meter
114 (F-22, HORIBA, Kyoto, Japan), we determined the pH (H_2O). We quantified Al and
115 heavy metals using inductively coupled plasma optical emission spectrometry
116 (ICP-OES; Optima 7300 V, PerkinElmer, Waltham, MA, USA), after digestion in
117 concentrated HNO_3-$HClO_4$ (1:4 v/v) at 140°C. Three replications of pH (H_2O) and
118 concentrations of total Al and heavy metals were conducted per collected root-zone soil
119 sample, for a total of 15 replications. The results for the 15 replications were averaged
120 and SEs were calculated. CEC, organic C, and total N of the root-zone soil were
121 determined in two replications each. CEC was determined according to the FAO method
122 (FAO 2014), and organic C and total N were determined with an NC analyzer
123 (Sumigraph NC-900, Sumika Chemical Analysis Service, Osaka, Japan).

124 Once a month from July 2012 to November 2012, one *M. sinensis* growing
125 within root-zone soil (200 × 200 × 200 mm volume) was arbitrarily collected per
126 sampling point, and five *M. sinensis* were collected per month. The collected samples
127 were washed with deionized water to remove soil particles (Gerke *et al*. 1994; Frérot *et*
128 *al*. 2006), and separated into above-ground parts, dead leaves, rhizomes, adventitious
129 roots, and flowers (flowers were present only from September to November). The

separated tissues were dried at 80°C for 48 h, ground, and pyrolyzed in concentrated HNO_3 at 130°C. The concentrations of Al and heavy metals (Cu, Fe, Pb, and Zn) except for Cd and Ni, which were lower in the root-zone soil than in an unpolluted Cambisol (Asami 2001), were determined in the plant tissues by ICP-OES. The concentrations in each tissue of the five *M. sinensis* per month were averaged and SEs were calculated.

Analysis of phenolic acids and organic acids in adventitious roots of mature M. sinensis

In September 2013, adventitious roots of mature *M. sinensis* were collected from the five sampling points and washed with deionized water. The roots were cut into pieces with scissors in methanol for extraction of phenolic acids or in 80% ethanol for extraction of organic acids. Samples were extracted for 1 week at 20°C in the dark.

The methanol extract was filtered and concentrated *in vacuo* at approximately 40°C. The concentrate was dissolved with 600 mL of water and extracted three times with ethyl acetate (200 mL each) to obtain the water and ethyl acetate phases. The water phase and ethyl acetate phase equivalent to 200 mg fresh weight (FW) of roots was dried *in vacuo* and then dissolved in 1 mL of 50% methanol, respectively. For phenolic acids, the resultant solution (10 μL) was analyzed by high-performance liquid chromatography (HPLC; Prominence UFLC series, Shimadzu, Kyoto, Japan) with analysis of spectral characteristics by means of a diode array detector (SPD-M20A; Shimadzu, Kyoto, Japan) according to the method described by Yamaji & Ichihara (2012). The results of three replications were averaged and SEs were calculated.

From the 80% ethanol extract, the water phase and ethyl acetate phase were obtained as described above. Organic acids were not detected in the ethyl acetate phase.

154 The water phase equivalent to 300 mg FW of roots was dried *in vacuo* and dissolved in
155 50% methanol. Then, the resultant solution was applied to a Toyopearl 650M column
156 (100 mm length × 14 mm i.d.; Tosoh Corporation, Yamaguchi, Japan), and organic acids
157 were eluted with 6 M formic acid. The eluate was freeze-dried (VD-250F; Taitec,
158 Saitama, Japan) to remove formic acid, and the residue was dissolved in 50 μL of
159 pyridine. Then, 100 μL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA;
160 Thermo Scientific, Bellefonte, PA, USA) was added, and the sample was
161 trimethylsilylated at 37°C for 30 min. We measured organic acid concentration by
162 means of gas chromatography-mass spectrometry (GC-MS) on a GC/MS-QP2010
163 instrument equipped with a GC-2010 electron-ionization mass spectrometry detector
164 (Shimadzu, Kyoto, Japan) and a low-polar InertCap 5MS/Sil capillary column (30 m ×
165 0.25 mm i.d., 0.25-μm film thickness; GL Sciences Inc., Tokyo, Japan) following
166 methods described by Yamaji *et al*. (2016b). Results for three replications were
167 averaged and SEs were calculated.
168

169 ## *Isolation of root endophytes from adventitious roots*

170 In August 2012, adventitious roots were collected and used for isolation of root
171 endophytes. In terms of ecological correspondence for plants and root endophytes
172 (Verheyen *et al*. 2003), fungi were isolated from the roots of plants, which were
173 simultaneously collected with root-zone soil. The adventitious roots were
174 surface-sterilized with 70% ethanol for 1 min, 2.25% hydrogen peroxide solution for 5
175 min, and 70% ethanol for 1 min. Then, they were rinsed twice with sterile deionized
176 water for 5 min to remove reagents, and were dried on sterile filter paper on a clean
177 bench for 5 min. The axenic roots were cut into approximately 10-mm segments with an

sterile scalpel: specifically, 100 segments were randomly cut from each of five individual *M. sinensis*. The 500 segments were placed on 1% malt extract agar medium (1% MA) and incubated at 23°C in the dark for 2 weeks. The isolated fungal species were microscopically observed (Olympus CX21, Olympus, Tokyo, Japan), and purified. The root endophyte detection percentage (%) for each fungus was calculated by means of the following formula:

Root endophyte detection percentage (%) = $(N_d/N_t) \times 100$

where N_d was the number of root segments from which the fungus was detected and N_t was the total number of root segments used for fungal isolation. Genera of frequently isolates were identified due to morphological observation.

Evaluation of Al- and Fe-chelating activities by means of CAS assays

In order to select root endophytes, which would interact chemically with *M. sinensis*, the Al- and Fe-chelating activities of the five most frequently isolated genera (*Chaetomium* spp., *Phialocephala* spp., *Lachnum* spp., and *Colletotrichum* spp.) were determined by assays on CAS-Al and CAS-Fe agar media. CAS-Al agar medium was prepared by the procedure for preparing CAS-Fe agar medium described by Alexander & Zuberer (1991), except that $AlCl_3 \cdot 6H_2O$ (10 μM) was used instead of $FeCl_3 \cdot 6H_2O$ (10 μM). Four isolates were randomly selected from each genus of root endophytes and the mycelial disk (5.5 mm i.d.) was placed on a CAS-Al or CAS-Fe agar plate (90 mm i.d.). After a 7-day incubation at 23°C in the dark, mycelial diameters at right angles to each other were measured and averaged, and clear zone diameters were measured in the same way. We evaluated the activity of each isolate by means of the following formula:

Al- or Fe-chelating activity = (clear zone diameter - colony diameter)/(colony diameter)
Results for three replications were averaged and SEs were calculated. *Chaetomium* species showed high Al- and Fe-chelating activity; therefore, the activity of all 38 isolates of *Chaetomium* were determined. Isolate 5R-7, which showed the highest activity, was used for inoculation tests, and was identified by means of morphological characteristics and molecular analysis. DNA templates were prepared from a small piece of mycelial mass, crushed in 50 µL sterilized water, and heated for 15 s in a microwave oven. ITS regions were amplified with primers ITS5 and ITS4 (White *et al*. 1990). PCR conditions included an initial denaturing step at 94°C for 4 min, 35 cycles at 94°C for 30 s, 52°C for 50 s, and 72°C for 50 s, and a final elongation at 74°C for 6 min. The reaction mixture included 25 µL of GoTaq master mix (Promega Co., Ltd. Wisconsin, USA), 10 pmol of each primer, and 1 µL of DNA template. Amplicons were purified with QIAquick PCR purification Kit (Qiagen, Hilden, Germany), sequenced with a BigDye Terminator cycle sequencing FS ready Reaction Kit ver. 3.1, and analyzed using the ABI3100 genetic analyzer (Applied Biosystems, California, USA). Obtained ITS sequences were deposited in the DNA Data Bank of Japan (accession No. LC188887). For molecular identification, the sequences were subjected to blast comparisons in the database of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Analysis of indole-3-acetic acid production by C. cupreum

Three mycelial disks (5.5 mm i.d.) of *C. cupreum* were inoculated in a 50-mL Erlenmeyer flask containing 15 mL of sterilized Czapek broth statically at 23°C in the dark for 12 days. After incubation, the medium was filtered, and the indole-3-acetic acid

(IAA) concentration was analyzed via measurement of absorbance at 530 nm with a UV-Vis spectrometer (UV-2450, Shimadzu, Kyoto, Japan) according to the procedure described by Gordon & Weber (1951). Results for three replications were averaged and SEs were calculated.

Soil-inoculation test with C. cupreum

Sterilization of root-zone soil and its properties

In November 2013, we collected the root-zone soil for inoculation test, because the inoculation test needed high amount of the root-zone soil. Dried and sieved (<2 mm) root-zone soil was sterilized by intermittent irradiation with 30-kGy γ-ray. The pH (H_2O) and the concentrations of available P, exchangeable Al, Ca, K, Mg, Na, Cu, Pb, and Zn were measured to evaluate the effect of γ-ray sterilization on the chemical properties of the soil. Exchangeable Al was extracted with 1 M KCl for 30 min. Exchangeable Ca, K, Mg, and Na were extracted with 1 M CH_3COONH_4 (20 mL, pH 7.0) at 100 rpm for 1 h. Available P was extracted with 1 mM H_2SO_4 at 100 rpm for 1 h. Exchangeable Cu and Zn were extracted with 0.1 M HCl at 100 rpm and 30°C for 1 h. Exchangeable Pb was extracted with 0.05 M $Ca(NO_3)_2$ at 100 rpm and 30°C for 1 h. For all the measurements, results of three replications were averaged and SEs were calculated. γ-ray sterilization of the soil affected the concentrations of available P, exchangeable Pb and Zn, and the pH (H_2O), although the differences were small, except in the case of available P (Table 1).

Preparation of mycelial solution and axenic seedlings

Chaetomium cupreum (isolate 5R-7), which exhibited high Al- and

Fe-chelating activities, was used for soil-inoculation tests. Twenty mycelial disks (5.5 mm i.d.) of *C. cupreum*, cut from the edge of the mycelia, were inoculated in a 300-mL Erlenmeyer flask containing 100 mL of 1% malt extract liquid medium with shaking at 23°C in the dark for 12 days. After incubation, the mycelia were rinsed with sterile deionized water to remove the medium. Then, the mycelia were homogenized with a homogenizer (HG-200, AS ONE Corporation, Osaka, Japan), and sterile deionized water was added to prepare a mycelial solution containing 6 mg of mycelial dry weight (DW) per milliliter. Sterile deionized water was used as a control.

The axenic seedlings of *M. sinensis* were prepared. Seeds were collected at the study site in November 2013 and were stored at 4°C until use. After seeds were soaked in deionized water for 5 min, the seeds were surface-sterilized with 70% ethanol for 2 min, 7.5% hydrogen peroxide solution for 5 min, and 70% ethanol for 2 min. Then, the seeds were rinsed twice with sterile deionized water for 5 min to remove reagents. The axenic seeds were incubated on 1/3 Hoagland medium containing 1.5% agar for 2 weeks (14 h light at 25°C/10 h dark at 20°C) in a growth chamber (NK Systems LP-100S, Nippon Medical & Instruments Co., Osaka, Japan). Germination started after 3 days of incubation, and seedlings at the second leaf stage were used for the inoculation tests.

Growth condition

On a clean bench, sterile root-zone soil (8 g) was transferred to a sterilized culture tube (AGC Techno Glass Co., Shizuoka, Japan; 120 mm height × 30 mm i.d.), which was covered with a polypropylene cap (AGC Techno Glass Co.). Then, sterile deionized water (8 mL) was added. One axenic seedling was aseptically transplanted

into each culture tube, and the tube was inoculated with the *C. cupreum* mycelial solution (2.4 mg of mycelial DW per 400 µL). Seedlings were grown in a growth chamber for 36 days (14 h light at 25°C/10 h dark at 20°C). The following two treatments were prepared: (1) seedlings with sterile deionized water as control and (2) seedlings inoculated with *C. cupreum* mycelial solution. Eleven seedlings (two seedlings for re-isolation, two seedlings for microscopic observation, three seedlings for element analysis with ICP-OES, and two seedlings each for analysis of phenolic acids or organic acids) were used per replication, and replications were conducted four times. Root length, FW of above-ground parts and roots, and DW of above-ground parts were measured for 11 seedlings per replication, in total 44 seedlings for each treatment. Root DW and water content of roots were measured for three seedlings per replication, in total 12 seedlings per treatment. After the inoculation test, inoculants from roots were re-isolated, and trypan-blue-stained roots were observed microscopically. For re-isolation and observation of trypan-blue-stained roots, two seedlings per replication, in total eight seedlings per treatment, were used. Our 36-day inoculation test was accomplished without contamination, as indicated by re-isolation of *C. cupreum* from the inoculated roots and no isolation from control roots.

Analysis of elements in plant tissues

Three seedlings per replication, in total 12 seedlings per treatment, were used for ICP-OES analysis. The above-ground parts were rinsed with deionized water. The roots were washed with 0.5 mM $CaCl_2$ (20 min × 3 times) to remove Al on their surface, and rinsed with deionized water. Above-ground parts and roots were dried and pyrolyzed as describe above. The concentrations of Al, Ca, K, Mg, Na, and P and the

heavy metals Cu, Fe, Pb, and Zn were determined. Results for 12 seedlings were averaged and SEs were calculated.

Analysis of phenolic acids and organic acids

The roots of two seedlings per replication, in total roots of eight seedlings per treatment, were extracted with methanol or with 80% ethanol for 24 h. The extracts were analyzed by HPLC or by GC-MS as describe above. Results for eight seedlings were averaged and SEs were calculated.

Localization of Al in seedling roots in water culture inoculation test

Growth condition

A culture tube lined with a strip of Advantec B51 chromatography paper (30 × 100 mm) was covered with a polypropylene cap, and autoclaved for 20 min. On a clean bench, 8 mL of sterile 1/10 Hoagland culture solution (pH 4.0) containing 100 μM $AlCl_3 \cdot 6H_2O$ was added to culture tube, and one axenic seedling was placed between the tube wall and the chromatography paper. A preliminary experiment using 0, 100, 250, and 500 μM $AlCl_3 \cdot 6H_2O$ indicated that *M. sinensis* could grow in 100 μM $AlCl_3 \cdot 6H_2O$, and Al did not precipitate. Two treatments were prepared as follows: (1) seedlings treated with a 1% MA disk (5.5 mm i.d.) as a control, and (2) seedlings inoculated with a mycelial disk (5.5 mm i.d.) of *C. cupreum* (isolate 5R-7) close to the root. Four seedlings were used per replication, and each replication was conducted four times. After inoculation for 36 days (14 h light at 25°C/10 h dark at 20°C) in a growth chamber, root length, FW of above-ground parts and roots, and DW of above-ground

parts and roots were measured for two seedlings per replication, in total, eight seedlings per treatment. Al concentrations in roots of the eight seedlings were quantified by ICP-OES, as described above. One seedling per replication, in total four seedlings per treatment, were used for re-isolation of *C. cupreum* from roots. One seedling per replication, in total four seedlings per treatment, were used for microscopic observation of trypan-blue-stained roots and Al localization.

Staining Al with lumogallion and observation by confocal laser microscopy and quantification of Al

Roots used for lumogallion staining were embedded in 4% agar gel, and a microtome (REM-710, Yamato Kohki Industrial Co., Saitama, Japan) was used to prepare 100-µm thick longitudinal sections. The sliced roots were stained with 10 µM lumogallion in 0.1 M sodium acetate buffer (pH 5.2) for 60 min at 50°C and were then washed with the buffer solution (15 min × 2 times) (Kataoka *et al*. 1997). The fluorescence emitted from the lumogallion-Al complex in the roots was observed by confocal laser microscopy (Fluoview; FV1000-D, Olympus, Tokyo, Japan) at an excitation wavelength of 473 nm, and the emission spectra were monitored at 520 nm (Kataoka *et al*. 1997). Al in roots was also quantified by ICP-OES, as described above, using two seedlings per replication, for a total of eight seedlings per treatment.

Statistical analysis

Statistical analysis was conducted with SPSS statistics software for Windows (ver. 22.0.0.0, IBM, Armonk, NY, USA). The differences in concentrations of available P, exchangeable Al, Ca, K, Mg, Na, and heavy metals (Cu, Pb, and Zn), and the pH

(H_2O) values of non-sterilized and γ-ray-sterilized soils were evaluated by Student's *t*-test. In soil-inoculation tests, between-treatment differences in seedling growth variables, water content of roots, and concentrations of heavy metals (Cu, Fe, Pb, Zn) and other inorganic elements (Al, Ca, K, Mg, Na, P) were evaluated by Student's *t*-test. For observations of Al, between-treatment differences in seedling growth were evaluated by Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

The properties of root-zone soil and concentrations of elements in mature M. sinensis roots

The properties of the root-zone soil are listed in Table 2, and the Al, Cu, Fe, Pb, and Zn concentrations in *M. sinensis* tissues are listed in Table 3. Although the concentrations of total Cu, Pb, and Zn were higher in the root-zone soil than in unpolluted Cambisol (Table 2) (Asami 2001), *M. sinensis* did not contain high concentrations of these heavy metals through the sampling period. The adventitious roots of *M. sinensis* contained high concentrations of Fe (exceeding 1000 mg kg^{-1} DW) through the sampling period, except for October (Table 3). The root-zone soil was acidic, and the exchangeable Al concentration was higher than that in unpolluted Cambisol (Table 2) (Ministry of the Environment 1999). The Al concentrations in the roots of *M. sinensis* exceeded 2000 mg kg^{-1} DW through the sampling period, except for October (Table 3).

Phenolic acids and organic acids in adventitious roots of mature

M. sinensis

In the adventitious roots of mature *M. sinensis*, we detected chlorogenic acid, malic acid, citric acid, and *trans*-aconitic acid as the Al detoxicants at concentrations of 0.13 ± 0.0, 0.14 ± 0.0, 0.16 ± 0.0, and 0.11 ± 0.0 mg g^{-1} FW, respectively.

Root endophytes isolation, CAS-Al and -Fe assays, and IAA production by C. cupreum

The five genera of root endophytes frequently isolated from adventitious roots of *M. sinensis* were *Chaetomium* (detection percentage: 9.2%), *Phialocephala* (8.2%), *Lachnum* (6.3%), and *Colletotrichum* (6.0%). The Al- and Fe-chelating activities of these fungal genera (four isolates per genus) were determined by means of CAS-Al and -Fe assays, and only *Chaetomium* isolates showed high Al- and Fe-chelating activities. Therefore, we preciously determined the Al- and Fe-chelating activities of all the isolates of *Chaetomium* (Table 4). We found that isolate 5R-7 showed the highest activities among 38 isolates, and DNA analysis revealed that isolate 5R-7 was *C. cupreum*. This isolate was deposited in the National Institute of Technology and Evaluation (deposit No. 111720) and in the author's (TH) private culture collection.

We investigated IAA production by *C. cupreum* (5R-7) and found that *C. cupreum* produced 3.81 ± 0.04 μg mL^{-1} of IAA. *Chaetomium cupreum* was used for the inoculation test to investigate its effects on the growth and Al tolerance in *M. sinensis*.

Effects of C. cupreum on M. sinensis seedling growth, and concentrations of elements and Al detoxicants in plant cells under

the soil-inoculation test

In order to clarify the chemical effect of *C. cupreum* on Al tolerance mechanisms in *M. sinensis* via chemical analyses, we conducted the soil-inoculation test using *C. cupreum* and *M. sinensis* seedlings. Although microsclerotia, which are fungal structures formed by endophytic fungi in plant cells, were not observed, mycelia growing along the epidermal cells were observed.

Both control seedlings and *C. cupreum*-inoculated seedlings did not show any symptoms of metal toxicities, such as growth inhibition, browning of roots, and chlorosis on leaves (Fig. 1). *Chaetomium cupreum* enhanced *M. sinensis* seedling growth in acidic and heavy metal-polluted soil (Table 5 and Fig. 1). Root length, FW of above-ground parts and roots, and DW of above-ground parts were significantly increased by inoculation of *C. cupreum* ($P < 0.001$, Table 5). *Chaetomium cupreum* also significantly increased DW of roots ($P < 0.01$, Table 5). Conversely, differences in the water content of roots were not statistically significant between treatments (Table 5).

Chaetomium cupreum did not enhance uptake of macro nutrient elements in the above-ground parts and roots (Fig. 2a and 2b). In the above-ground parts, *C. cupreum* significantly decreased Cu and Pb concentrations ($P < 0.05$, Fig. 3a). However, Fe concentration in the above-ground parts was significantly higher in seedlings inoculated with *C. cupreum* than in non-inoculated seedlings ($P < 0.05$, Fig. 3a). In roots, Al, Cu, Fe, and Pb concentrations were significantly higher in seedlings inoculated with *C. cupreum* than in non-inoculated seedlings ($P < 0.05$, Fig. 3b). In roots, we detected chlorogenic acid, malic acid, citric acid, and chlorogenic acid derivative, which are Al detoxicants (Table 6). The concentrations of malic acid and citric acid were significantly lower in seedlings inoculated with *C. cupreum* than in non-inoculated seedlings (for

malic acid, $P < 0.001$; for citric acid, $P < 0.01$, Table 6).

Effects of C. cupreum on Al localization in M. sinensis seedlings roots in water culture inoculation test

In the presence of Al, root length and the DW of roots were significantly increased in seedlings inoculated with *C. cupreum* ($P < 0.01$, Table 7 and Fig. 4). Control roots and inoculated roots with *C. cupreum* contained 1770.6 ± 265.0 mg kg^{-1} DW and 962.20 ± 153.09 mg kg^{-1} DW of Al, respectively. Both control seedlings and *C. cupreum*-inoculated seedlings did not show any symptoms of Al toxicity, such as growth inhibition, browning of roots, and chlorosis on leaves (Fig. 4).

Localization patterns of Al in the root cross-sections were different between treatments (Fig. 5). In sections of non-inoculated roots, fluorescence of lumogallion-Al complex was observed throughout the section (Fig. 5b). In contrast, in sections of inoculated roots, fluorescence of lumogallion-Al complex was observed primarily in cell walls of the stele (Fig. 5d). Moreover, fluorescence of lumogallion-Al complex was also observed in the mycelia of *C. cupreum* growing on the surface of the roots (Fig. 6).

Discussion

Miscanthus sinensis grows naturally at the Hitachi mine, which contains high concentrations of Al owing to the acidity of the soil, and high concentrations of heavy metals (Table 2). The concentrations of exchangeable Al, Cu, Pb, and Zn in the root-zone soil were higher compared with those in unpolluted Cambisol [for Al see (Ministry of the Environment 1999); for Cu, Pb, and Zn see (Asami 2001)] (Table 1).

Therefore, bioavailable Al and heavy metals would be high in the root-zone soil and *M. sinensis* should be able to absorb Al and heavy metals easily. However, the concentrations of Cu, Pb, and Zn in all tissues of *M. sinensis* were low (Table 3). Conversely, the Al concentration in the adventitious roots (above 2000 mg kg^{-1} DW) (Table 3) was remarkably high compared with that reported in previous studies on Al content in other plant tissues (Hutchinson 1945; Robinson & Edgington 1945; Chenery 1948). This result indicates that *M. sinensis* should have Al tolerance mechanisms. Actually, HPLC and GC-MS analysis verified that adventitious roots of mature *M. sinensis* produced chlorogenic acid, malic acid, citric acid, and *trans*-aconitic acid, which are known to detoxify toxic Al (Nagata *et al*. 1992; Pellet *et al*. 1995; Adams *et al*. 2002). From these results, we infer that mature *M. sinensis* acquires Al tolerance via production of Al detoxicants, as one of the tolerance mechanisms.

Next, we verified the Al tolerance mechanism in the plant in association with root endophytes. The major root endophyte, *Chaetomium* isolates, showed high siderophore production, as indicated by CAS-Al and CAS-Fe assays (Table 4), and this result indicated that *Chaetomium* can detoxify Al by producing siderophores. Although *Chaetomium* species are well known to produce various antifungal compounds, resulting in protection of the host plants (Soytong *et al*. 2001; Li *et al*. 2011b), there was no reports of *Chaetomium* species to produce siderophores. Among the *Chaetomium* isolates, *C. cupreum* (5R-7), which exhibited the highest siderophore production, was used in the soil-inoculation test using mine soil. Compared with control seedlings, *C. cupreum* inoculation significantly promoted seedling growth (Table 5), probably via production of IAA, but not enhancing uptake of nutrient elements (Fig. 2). Both control roots and *C. cupreum*-inoculated roots contained high concentrations of Al (Fig 3b),

which would show Al toxicity to many plant species (Kabata-Pendias 2011); however, all the roots grew well and showed no symptoms of Al toxicity, such as inhibition of root growth (Ryan *et al*. 1993; Kochian *et al*. 2005). Using the concentrations of Al detoxicants in found in the roots (Table 6), we calculated the Al concentration that could be detoxified by means of a previously reported method (Barceló & Poschenrieder 2002). Control seedlings could detoxify approximately 1400 mg kg^{-1} DW of Al in roots, whereas seedlings inoculated with *C. cupreum* could detoxify only 280 mg kg^{-1} DW of Al in roots. This result indicated that the concentrations of Al detoxicants produced by *M. sinensis* were not sufficient to detoxify all the Al in the control roots and *C. cupreum*-inoculated roots. Other mechanisms of Al tolerance, except for the detoxicants production, must be considered in *M. sinensis* seedlings.

To determine other Al-tolerance mechanisms in *M. sinensis*, Al localization in root cells and mycelia was observed using water culture inoculation test. In control roots, Al was localized in cell walls throughout the root cross-section (Fig. 5b) and Al appeared to be detoxified by localization in cell walls, which contributes to the intrinsic Al tolerance in plants (Vögeli-Lange & Wagner 1990). *Chaetomium cupreum* changed the Al localization pattern in root cells compared with that of the control (Fig. 5d). Al was present at particularly high levels in cell walls of the epidermis, endodermis, and stele of roots. The endodermis has a casparian strip, which limits movement of harmful elements and substances from the cortex to the stele (MacFarlane & Burchett 2000). Therefore, *C. cupreum* enhanced Al detoxification via a more functional localization pattern of Al in *M. sinensis.* Additionally, *C. cupreum* accumulated Al in the external mycelia growing on the surface of roots (Fig. 6). This result suggests that *C. cupreum* enhanced Al tolerance in *M. sinensis* by compartmentalizing Al into mycelia. Moreover,

Al accumulation in fungal mycelia surrounding the roots might contribute to high concentration of Al in the roots of *M. sinensis* in the soil-inoculation test (Fig. 3b). Other studies have also reported that the accumulation of harmful elements into fungal mycelia via the production of siderophores (Rajkumar *et al*. 2012), as well as the changes in mineral localizations in plant cells stimulated by microorganisms (Wang *et al*. 2016). Our results indicated that *C. cupreum* conferred a more efficient Al-tolerance mechanism to *M. sinensis* via accumulating Al into mycelia and changing Al localization into more harmless tissues. In the next research, we should preciously investigate mycelial colonization pattern in the inoculated roots with *C. cupreum*, in order to elucidate the mechanisms such as mycelial stimulation to plant cells, for example.

From an ecological perspective, we point out that *M. sinensis* could be a pioneer species at mine sites (Hiroi, 1974). Furthermore, *M. sinensis* could be the first invader in volcanic regions and the sole plant species growing highly acidic soils (Yoshii, 1937; An *et al*. 2008). However, there is no report to clarify that *M. sinensis* growing in acidic mine site accumulates a high concentration of Al, not other heavy metals. Our study seems to indicate that *M. sinensis* appeared to acquire heavy-metal tolerance, inhibiting high absorption of heavy metals (Table 3); heavy metal tolerance in plants could be mainly categorized into two mechanisms, such as “exclusion mechanism” and “detoxification mechanism” (Larcher 2003). In the soil-inoculation test, both control roots and *C. cupreum*-inoculated roots contained high concentrations of Al and Fe, although the concentrations of Cu, Pb and Zn were low (Fig. 3b); although *C. cupreum* significantly enhanced higher uptakes of Cu and Pb in roots than control, the concentrations maintain too low to cause toxicities to many plant species previously

reported (Kabata-Pendias 2011). It has been reported that Al inhibits Cu uptake and Fe inhibits Zn uptake in roots (Kabata-Pendias 2011). Therefore, Al and Fe accumulation in *M. sinensis* might inhibit excessive Cu and Zn uptake, resulting in heavy-metal tolerance. Recently, some researches have shown that Al could be a useful element for some plant species: Al enhances photosynthesis in *Camellia sinensis* (Hajiboland *et al*. 2013) and promotes root growth in *Quercus serrata* (Tomioka *et al*. 2005). In *M. sinensis*, which can accumulate high concentration of Al via support from the root endophytic *C. cupreum*, Al might be beneficial for survival in acidic heavy-metal polluted environments.

In summary, we found that *M. sinensis* growing at the acidic mine site had Al tolerance and could detoxify Al intrinsically via the production of Al detoxicants and accumulation of Al in cell walls. In addition, *M. sinensis* had a more functional Al tolerance via support by the root endophytic *C. cupreum*; *C. cupreum*, which produces siderophores, reduced Al toxicity on *M. sinensis* via accumulating Al in mycelia surrounding the roots, and altering the Al-localization pattern in the plant roots, which was more efficient in the detoxification of Al. Furthermore, Al accumulation in *M. sinensis* might inhibit excessive heavy-metal uptake, and it might enable *M. sinensis* to be a pioneer species in acidic mine site. Finally, we suggest that root endophytes would be needed to make *M. sinensis* a more suitable plant for afforestation in acidic mine sites and other acidic denuded sites. We hope that our study contributes to practical studies on revegetation via providing basic knowle4dge about plant-microbe interactions.

Acknowledgments

The ICP, GC-MS analysis and observation of Al localization by confocal laser microscopy were carried out at the OPEN FACILITY, Research Facility for Science and Technology, University of Tsukuba. This work was supported by the Sasakawa Scientific Research Grant from The Japan Science Society.

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Figure legends

Fig. 1 *Miscanthus sinensis* seedlings after soil-inoculation test. (a) Image of *M. sinensis* seedling without *Chaetomium cupreum* (control seedling). (b) Image of *M. sinensis* seedling inoculated with *C. cupreum*. Scale bars represent 1 cm.

Fig. 2 Concentrations of nutrient elements in *Miscanthus sinensis* seedlings in the soil-inoculation test. Concentrations in (a) above-ground parts and (b) roots. Differences between inoculated seedlings with *Chaetomium cupreum* and non-inoculated seedlings (control seedlings) were evaluated by Student's *t*-test. Results are expressed as means ± SEs (n = 12). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

686 **Fig. 3 Concentrations of Al and heavy metals in *Miscanthus sinensis* seedlings in**
687 **the soil-inoculation test.** Concentrations in (a) above-ground parts and (b) roots.
688 Differences between inoculated seedlings with *Chaetomium cupreum* and
689 non-inoculated seedlings (control seedlings) were evaluated by Student's *t*-test. Results
690 are expressed as means ± SEs (n = 12). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

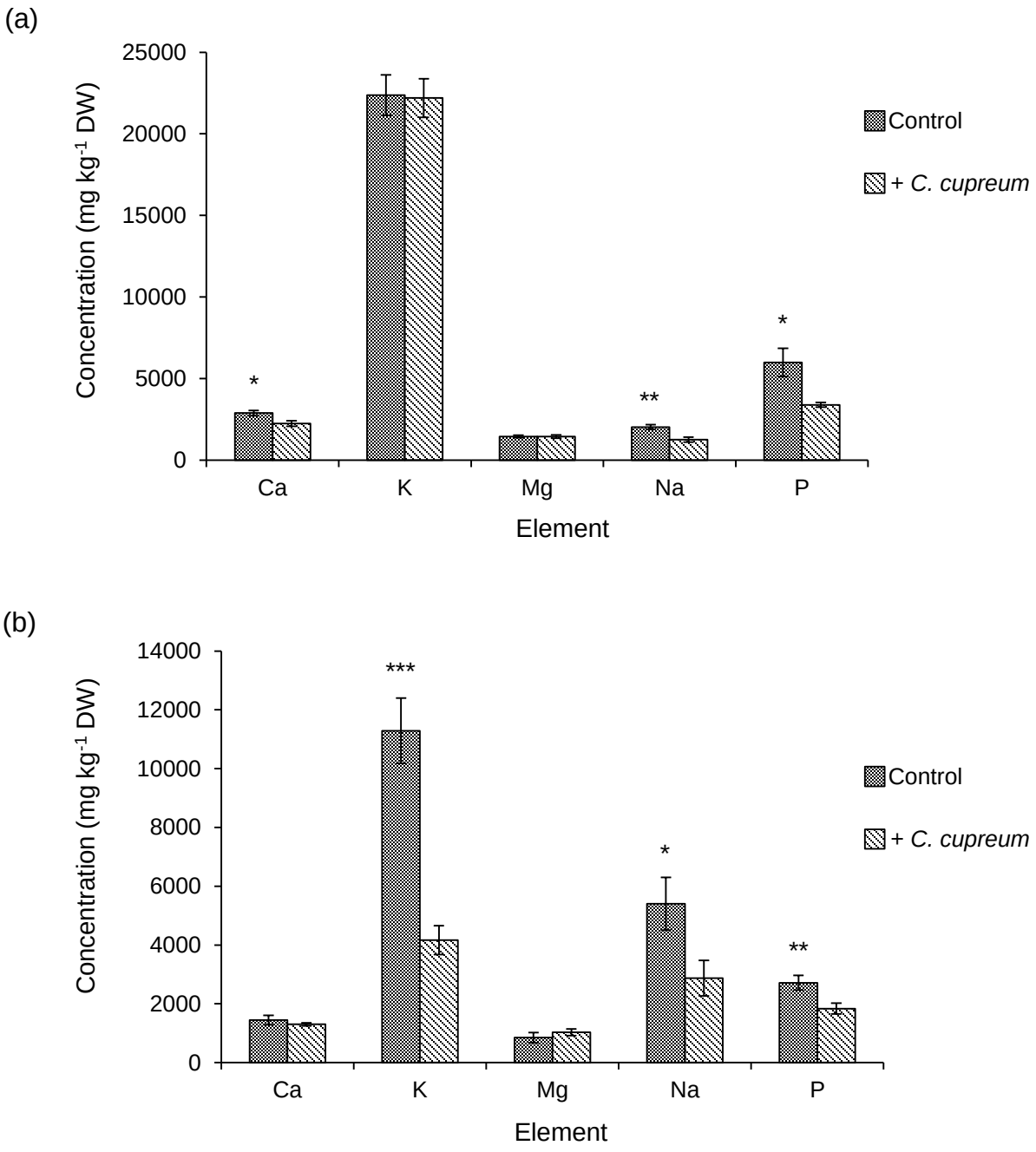
691 **Fig. 4 *Miscanthus sinensis* seedlings after water culture inoculation test.** (a) Image
692 of *M. sinensis* seedling without *Chaetomium cupreum* (control seedling). (b) Image of
693 *M. sinensis* seedling inoculated with *C. cupreum*. Scale bars represent 1 cm.

Fig. 5 Al localization in root sections of *Miscanthus sinensis* seedling roots in the water culture containing Al. (a) and (b) indicate root sections without *Chaetomium cupreum* (control seedling), and (c) and (d) indicate root sections with *C. cupreum*. (a) and (c) are bright light images observed using an optical microscope mode. (b) and (d) indicate fluorescence emitted from lumogallion-Al complex (green). S, stele; En, endodermis; Ep, epidermis. Scale bars represent 50 μm.

Fig. 6 Roots of *Miscanthus sinensis* seedlings stained with trypan blue or lumogallion. (a) Image of roots inoculated with *Chaetomium cupreum* in the presence of Al. Arrow indicates mycelia of *C. cupreum* stained with trypan blue. (b) Image of roots inoculated with *C. cupreum* in the presence of Al; overlay of fluorescence emitted from lumogallion-Al complex (green) and bright light images. Arrow indicates mycelia of *C. cupreum*, and green color indicates Al localization. Scale bars represent 50 μm.

Figure 1.

Figure 2.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Concentration (mg kg-1 DW)
*
**
*
Ca
K
Mg
Na
P
Element
Control
+ C. cupreum
(b)
14000
12000
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8000
6000
4000
2000
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*
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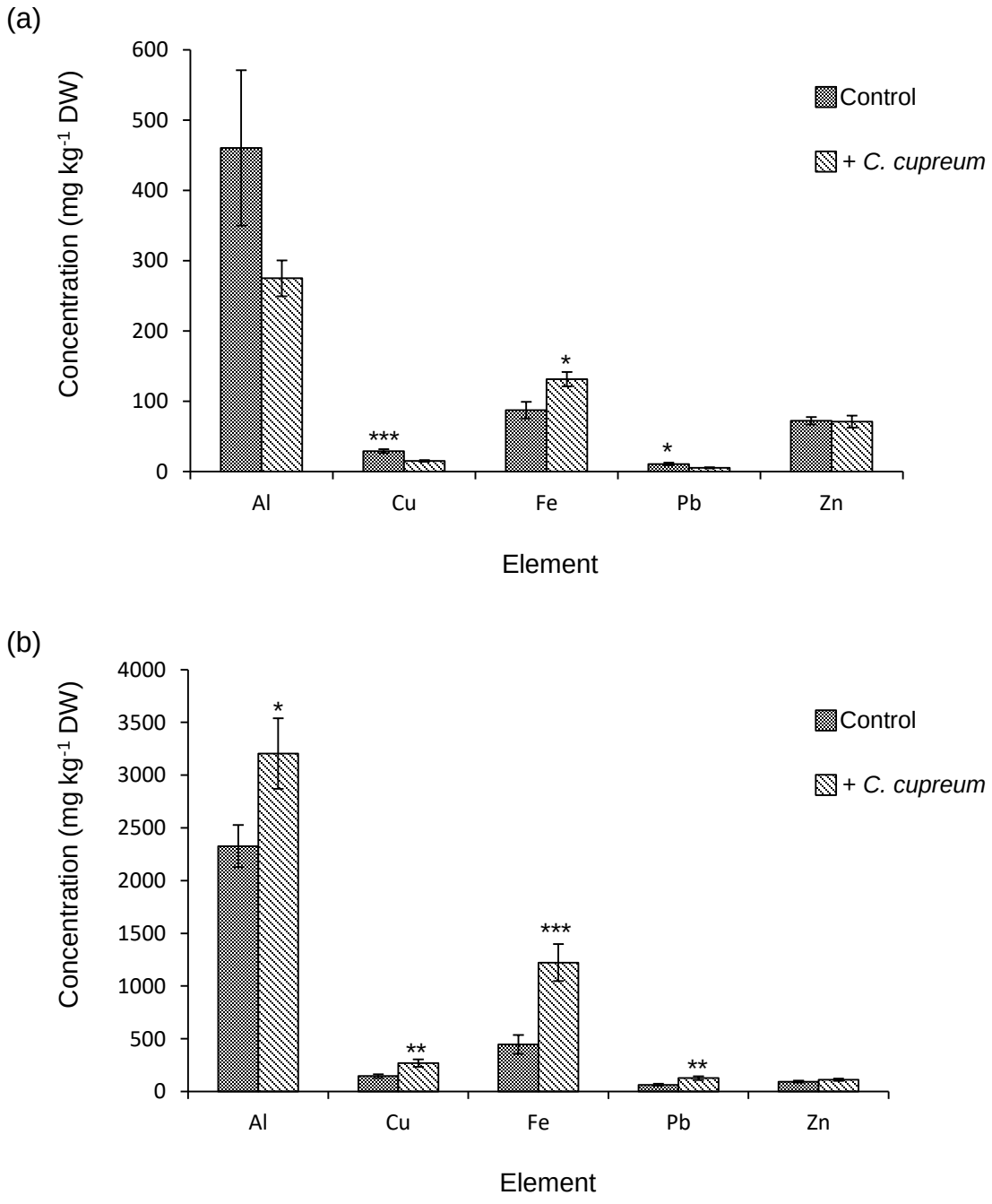
Ca
K
Mg
Na
P
Element
Control
+ C. cupreum

Figure 3.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Concentration (mg kg-1 DW)
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Pb
Zn
Element
(b)
4000
3500
3000
2500
2000
1500
1000
500
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Concentration (mg kg-1 DW)
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+ C. cupreum
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Pb
Zn
Element

Figure 4.

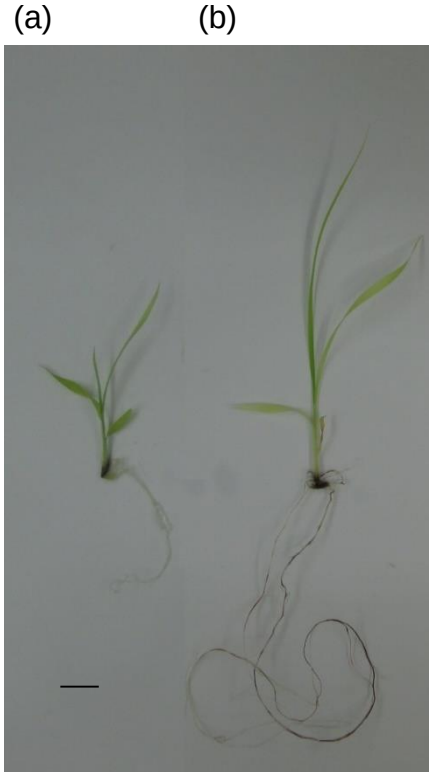


Figure 5.

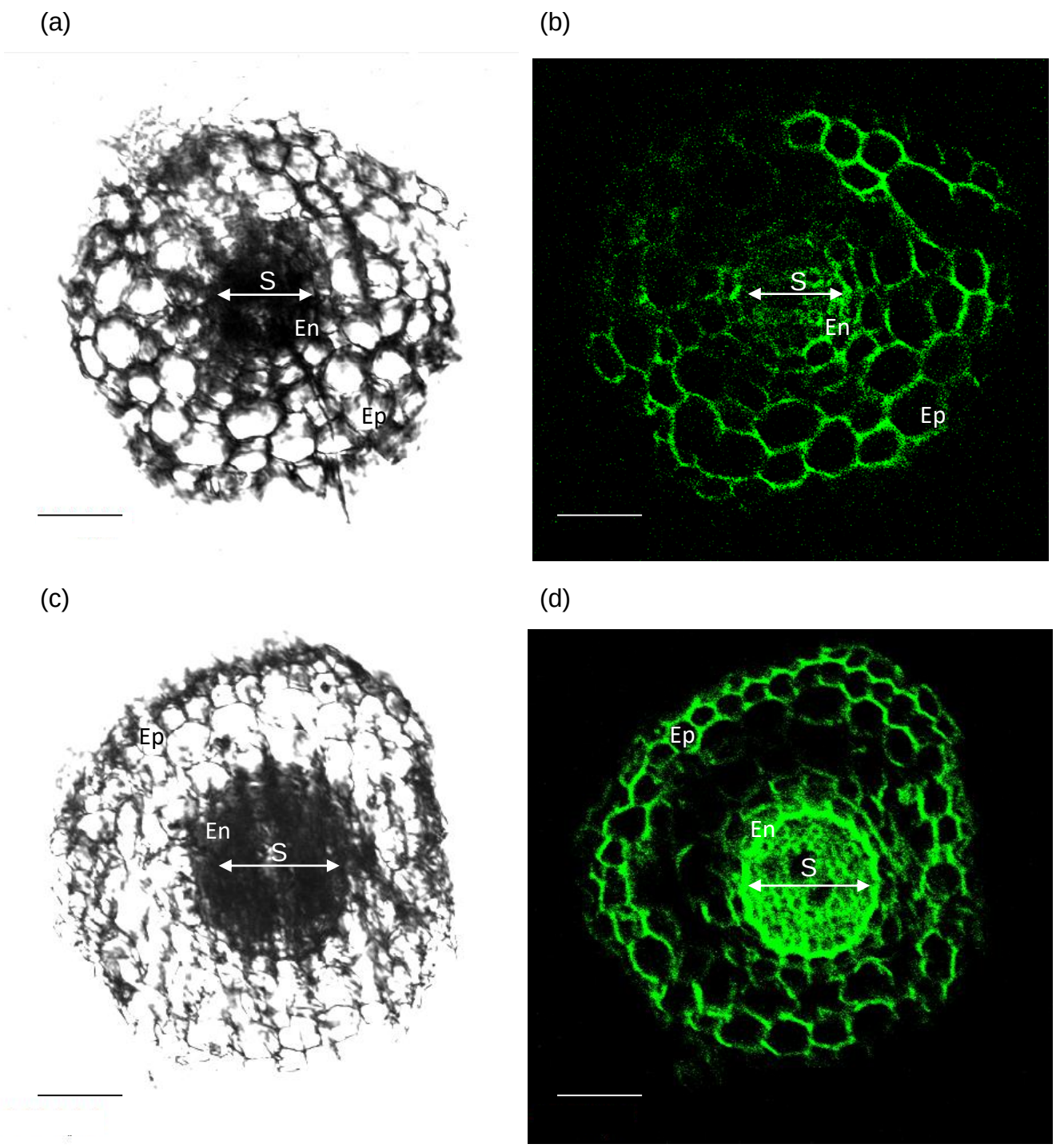
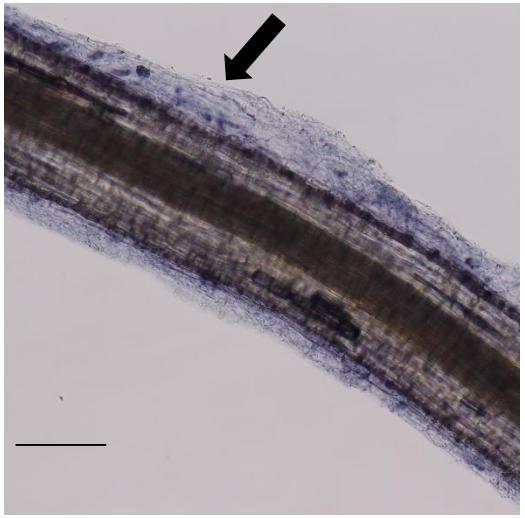


Figure 6.

(a)



(b)

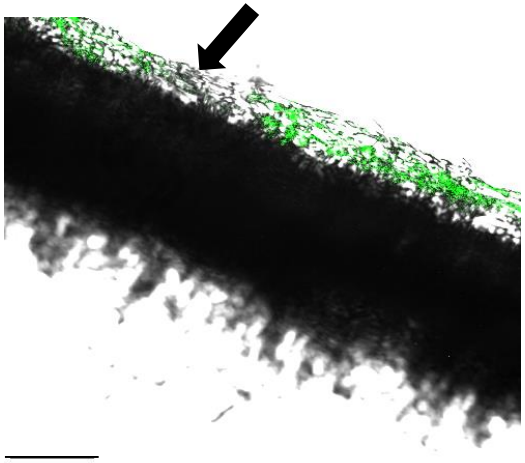


Table 1 Concentrations of available P, exchangeable Al, Ca, K, Mg, Na, and heavy metals, and pH (H_2O) values in non-sterilized and γ-ray-sterilized soils.

	Non-sterilized	Sterilized
pH (H_2O)	4.27 ± 0.01	4.23 ± 0.01*
Al (mg kg^{-1})	416.35 ± 17.25	445.12 ± 9.34
Ca (mg kg^{-1})	330.35 ± 5.65	336.14 ± 4.28
K (mg kg^{-1})	156.97 ± 5.54	157.51 ± 1.42
Mg (mg kg^{-1})	44.93 ± 0.82	46.57 ± 0.70
Na (mg kg^{-1})	19.86 ± 4.54	15.43 ± 0.41
P (mg kg^{-1})	8.34 ± 2.56	19.84 ± 2.03*
Cu (mg kg^{-1})	81.49 ± 2.11	81.44 ± 1.39
Pb (mg kg^{-1})	177.15 ± 1.51	185.38 ± 0.74**
Zn (mg kg^{-1})	13.35 ± 0.03	14.72 ± 0.38*

Results are expressed as means ± SEs (n = 3). **, $P < 0.01$; *, $P < 0.05$.

Table 2 Properties of root-zone soil.

Properties	Value
pH (H_2O)	4.4 ± 0.05
Total Al (mg kg^{-1})	54000 ± 1700
Total Cd (mg kg^{-1})	ND
Total Cu (mg kg^{-1})	440 ± 9.6
Total Fe (mg kg^{-1})	38600 ± 1010
Total Ni (mg kg^{-1})	15 ± 0.25
Total Pb (mg kg^{-1})	420 ± 22
Total Zn (mg kg^{-1})	83 ± 6.0
Cation exchange capacity [cmol (+) kg^{-1}]	90
Organic C (%)	13
Total N (%)	1.3

Al and heavy metal concentrations, and pH (H_2O) values are expressed as means ± SEs (n = 15). Cation exchange capacity, organic C, and total N are expressed as means (n = 2). ND indicates that the concentration was below the detection limit.

Table 3 Al and heavy metal concentrations in *Miscanthus sinensis* tissues, July–November.

(a) July

Element (mg kg^{-1} DW)	Above-ground parts	Dead leaves	Rhizomes	Adventitious roots
Al	284.3 ±39.1	989.64 ± 147.6	1852.1 ± 262.9	2099.3 ± 418.6
Cu	4.9 ± 0.4	19.2 ± 1.7	40.1 ± 4.6	84.8 ± 17.6
Fe	141.0 ± 40.0	643.5 ±103.6	1279.5 ± 277.7	1053.5 ± 252.2
Pb	3.4 ± 0.3	23.4 ± 1.3	89.2 ± 25.3	170.1 ± 32.9
Zn	58.5 ± 20.4	71.2 ± 21.2	261.1 ± 194.4	134.3 ± 61.3

(b) August

Element (mg kg^{-1} DW)	Above-ground parts	Dead leaves	Rhizomes	Adventitious roots
Al	83.3 ± 5.1	1025.5 ± 256.7	893.5 ± 296.7	2336.4 ± 451.1
Cu	7.7 ± 0.7	27.2 ± 4.3	21.4 ± 4.9	107.6 ± 7.0
Fe	ND	665.1 ± 183.9	474.2 ± 222.2	1183.1 ± 269.7
Pb	1.5 ± 0.5	21.6 ± 4.5	16.1 ± 5.3	103.3 ± 7.2
Zn	15.8 ± 6.0	14.6 ± 3.7	27.8 ± 5.5	81.5 ± 14.5

(c) September

Element (mg kg^{-1} DW)	Above-ground parts	Dead leaves	Rhizomes	Adventitious roots	Flowers
Al	95.4 ± 10.0	439.9 ± 191.3	1482.8 ± 272.1	2675.1 ± 417.2	69.3 ± 17.3
Cu	8.7 ± 0.1	18.8 ± 3.6	27.4 ± 3.6	78.1 ± 6.9	9.5 ± 1.2
Fe	64.4 ± 4.7	256.4 ± 43.8	857.3 ± 215.4	1402.7 ± 247.5	46.0 ± 5.7
Pb	5.7 ± 0.6	13.3 ± 1.0	32.2 ± 5.0	50.6 ± 6.1	1.8 ± 0.2
Zn	44.0 ± 9.6	36.3 ± 9.1	55.9 ± 10.3	33.0 ± 5.3	55.1 ± 10.6

(d) October

Element (mg kg^{-1} DW)	Above-ground parts	Dead leaves	Rhizomes	Adventitious roots	Flowers
Al	89.6 ± 6.3	292.6 ± 17.0	559.9 ± 119.4	1742.3 ± 182.6	61.1 ± 4.4
Cu	5.2 ± 0.9	11.2 ± 1.1	13.7 ± 3.3	74.1 ± 13.4	14.2 ± 2.0
Fe	44.1 ± 6.7	167.9 ± 9.7	300.8 ± 52.4	902.6 ± 87.7	13.0 ± 7.1
Pb	0.8 ± 0.4	3.4 ± 0.5	4.9 ± 1.7	35.8 ± 6.8	4.7 ± 0.9
Zn	26.5 ± 4.4	27.1 ± 3.2	40.4 ± 3.2	33.6 ± 4.3	35.3 ± 5.3

(e) November

Element (mg kg^{-1} DW)	Above-ground parts	Dead leaves	Rhizomes	Adventitious roots	Flowers
Al	70.0 ± 8.4	207.7 ± 22.7	448.1 ± 90.6	2288.6 ± 484.0	133.7 ± 55.5
Cu	4.5 ± 0.6	9.6 ± 0.6	16.4 ± 2.2	78.1 ± 9.3	18.8 ± 1.4
Fe	20.5 ± 3.8	109.7 ± 17.2	264.0 ± 60.7	1335.4 ± 329.8	83.4 ± 9.6
Pb	3.9 ± 0.7	9.5 ± 0.6	17.5 ± 2.4	87.3 ± 11.0	17.1 ± 1.2
Zn	77.8 ± 19.2	30.3 ± 2.9	51.3 ± 3.8	38.6 ± 6.7	47.3 ± 10.2

DW, dry weight. Results are expressed as means ± SEs (n = 5). ND indicates that the concentration was below the detection limit.

Table 4 Al- and Fe-chelating activities of *Chaetomium* isolates, as indicated by CAS-Al and CAS-Fe assays.

Isolates	CAS-Al	CAS-Fe	Isolates	CAS-Al	CAS-Fe
7R-1	2.0 ± 0.4	1.3 ± 0.2	8R-4	2.7 ± 0.1	1.5 ± 0.1
7R-2	-	1.1 ± 0.6	8R-5	1.8 ± 0.1	1.4 ± 0.1
7R-3	-	+	8R-6	1.9 ± 0.2	-
7R-4	0.3 ± 0.2	-	8R-7	-	-
7R-5	-	+	8R-8	0.6 ± 0.2	0.6 ± 0.1
7R-6	+	+	8R-9	1.8 ± 0.6	1.1 ± 0.1
7R-7	0.9 ± 0.5	0.6 ± 0.3	8R-10	-	0.9 ± 0.4
7R-8	0.2 ± 0.1	+	8R-11	0.6 ± 0.2	0.6 ± 0.1
7R-9	+	+	8R-12	0.3 ± 0.1	+
3R-1	1.4 ± 0.1	0.7 ± 0.1	8R-13	0.9 ± 0.5	-
3R-2	0.6 ± 0.4	1.0 ± 0.3	8R-14	0.5 ± 0.1	0.7 ± 0.2
3R-3	0.4 ± 0.1	0.5 ± 0.1	8R-15	0.8 ± 0.3	0.8 ± 0.5
3R-4	0.2 ± 0.2	-	5R-1	+	+
3R-5	-	+	5R-2	1.7 ± 0.8	1.4 ± 0.7
3R-6	+	0.4 ± 0.2	5R-3	-	+
3R-7	0.4 ± 0.1	0.4 ± 0.3	5R-4	2.3 ± 0.2	2.2 ± 0.1
8R-1	1.8 ± 1.0	-	5R-5	+	+
8R-2	3.0 ± 0.3	1.9 ± 0.1	5R-6	2.1 ± 0.3	2.6 ± 0.2
8R-3	3.6 ± 0.9	2.9 ± 0.4	5R-7	4.0 ± 0.4	3.0 ± 0.5

Results are expressed as means ± SEs (n = 3). + indicates that the clear zone diameter was approximately equal to the mycelial diameter, and - indicates that the clear zone diameter was less than the mycelial diameter.

Table 5 Seedling growth in soil-inoculation test.

Treatment	Root length (cm)	Above-ground parts FW (mg)	Roots FW (mg)	Above-ground parts DW (mg)	Roots DW (mg)	Water contents of roots (%)
Control	10.6 ± 0.8	24.2 ± 1.1	8.9 ± 0.5	4.2 ± 0.2	1.3 ± 0.1	85.8 ± 1.4
C. cupreum	43.9 ± 3.2***	36.0 ± 1.8***	22.0 ± 1.7***	7.3 ± 0.4***	2.7 ± 0.2**	89.5 ± 0.4

FW, fresh weight; DW, dry weight. Results are expressed as means ± SEs (n = 44, except for n = 12 for root DW and water content of roots). Differences between treatments were evaluated by Student's t-test. ***, $P < 0.001$; **, $P < 0.01$.

Table 6 Concentrations of Al detoxicants in roots of *Miscanthus sinensis* seedlings in soil-inoculation test.

Treatment	Chlorogenic acid (μg mg^{-1} FW)	Chlorogenic acid derivative (μg mg^{-1} FW)	Malic acid (μg mg^{-1} FW)	Citric acid (μg mg^{-1} FW)
Control	0.34 ± 0.16	ND	0.30 ± 0.07	0.47 ± 0.15
C. cupreum	0.02 ± 0.00	0.03 ± 0.01	0.04 ± 0.01***	0.07 ± 0.01**

FW, fresh weight. Results are expressed as means ± SEs (n = 8). Differences between treatments were evaluated by Student's t-test. ***, $P < 0.001$; **, $P < 0.01$. ND indicates that the concentration was below the detection limit.

Table 7 Seedling growth in water culture inoculation test.

Treatment	Root length (cm)	Above-ground parts FW (mg)	Roots FW (mg)	Above-ground parts DW (mg)	Roots DW (mg)
Control	21.1 ± 6.7	12.7 ± 1.6	10.1 ± 3.1	1.4 ± 0.3	0.7 ± 0.3
C. cupreum	50.5 ± 4.5**	14.2 ± 1.2	14.4 ± 1.3	1.9 ± 0.2	1.9 ± 0.1**

FW, fresh weight; DW, dry weight. Results are expressed as means ± SEs (n = 8). Differences between treatments were evaluated by Student's t-test. **, $P < 0.01$.