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Effect of citric acid and rhizosphere bacteria on metal plaque formation and metal accumulation in reeds in synthetic acid mine drainage solution

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ABSTRACT

Many of regions in the world have been affected by acid mine drainage (AMD). The study assessed the effect of rhizosphere bacteria and citric acid (CA) on the metal plaque formation and heavy metal uptake in *Phragmites australis* cultured in synthetic AMD solution. Mn and Al plaque were not formed, but Fe plaque which was mediated by rhizosphere iron oxidizing bacteria (Fe(II)OB) was observed on the root system of reeds. Fe plaque did not significantly influence the uptake of Fe, Al and Mn into tissues of reeds. CA significantly ($p < 0.01$) inhibited the growth of Fe(II)OB and decreased the formation of Fe plaque. CA also significantly improved ($p < 0.05$) the accumulation of Fe, Mn and Al in all the tissues of reeds. Roots and rhizomes were the main organs to store metals. The roots contained 0.08 ± 0.01 mg/g Mn, 2.39 ± 0.26 mg/g Fe and 0.19 ± 0.02 mg/g Al, while the shoots accumulated 0.04 ± 0.00 mg/g Mn, 0.20 ± 0.01 mg/g Fe, 0.11 ± 0.00 mg/g Al in reeds cultured in solution amended with 2.101 g/l CA and without inoculation of rhizosphere bacteria.

1. Introduction

Many of regions in the world have been affected by acid mine drainage (AMD) which is characterized by low pH, high concentrations of heavy metals such as Fe, Mn, Al and Cu, and high conductivity (Batty et al., 2000; Herlihy et al., 1990). Heavy metals enter the waters and soils and then become available and toxic to organisms and human beings. For instance, many benthic invertebrates were sensitive heavy metals such as Fe, Mn and Zn present in water and sediment (Dsa et al., 2008). Al was reported to cause acute toxicity to fish and to alter snail behavior though ingestion (Soucek et al., 2001). *Phragmites australis* which is known to be widely distributed in the world, be able to survive under acidic conditions and accumulate some heavy metals distinctly more than other wetlands plants, is commonly used to uptake and remove metals from AMD (Batty et al., 2000; Duman et al., 2007).

Metal plaque, such as iron (oxy-)hydroxide and manganese oxide, have been widely observed around the root system of wetland plants grown in mine drainage (Batty et al., 2002). Studies have indicated that plant and rhizosphere microbial systems can affect metal plaque formation on roots of plants (Neubauer et al., 2007). Metal plaque may sequester metals by adsorption or co-precipitation, and thus affect the availability of elements and their accumulation and translocation in plants (Liu et al., 2010). However, the exact role played by metal plaque has had conflicting reports. Some studies indicated that metal plaque may act as a "barrier" and decrease metal accumulation in plants (Chen et al., 2005). Others indicated that metal plaque may increases the accumulation of essential and nonessential metals (Zhang et al., 1998). It was also reported that chelators, such as citric acid (CA), can affect metal bioavailability for plants, depending upon the molecular weight of the formed metal-chelate complexes, the surrounding pH, etc. (Taylor et al., 1984; Macfie and Crowder, 1987). However, there has been very little information pertaining to the interactions between CA and the growth of rhizosphere microorganisms, which also influence metal bioavailability and metal uptake in plant tissues (Kuffner et al., 2008).

The overall objective of this study was to assess the effect of CA and rhizosphere bacteria on the metal plaque formation and metal uptake in *P. australis* cultured in synthetic acid mine drainage solution. A hydroponic experiment was conducted for two reasons. First, *P. australis* was selected as it is a typical wetland plant which can grow in aquatic system (Duman et al., 2007; Baldantoni et al., 2009). Second, hydroponic experiments have less mass transfer limitations than soils and therefore yield better understanding of the fate of metals in solution and plants (Asao, 2012). The specific aims were to (1) assess the role of rhizosphere bacteria in metal

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plaque formation on the root system of reeds; (2) investigate the role of CA on the growth of rhizosphere bacteria and metal plaque formation; and (3) determine the effect of metal plaque and CA on element uptake in reeds.

2. Materials and methods

2.1. Plant sources

Rhizomes of reeds were collected from an AMD contaminated site located in Beaver Township, North Lima, OH, USA. The rhizomes were then transferred into commercial potting soil (Micracle-Gro lawn products, Inc) in pans (45 cm × 25 cm × 7.5 cm) and cultured in a greenhouse. The average temperature of the greenhouse was 22 °C and the humidity was 50 percent. After 30 days of growth, new roots and shoots propagated from the rhizomes. Seedlings (including original rhizomes, new roots and shoots) with similar biomass were used to initiate hydroponic experiments.

2.2. Hydroponic solutions

In hydroponic experiments, the control group only contained nutrient materials which included 0.250 g N (NH_4NO_3), 0.060 g Mg ($MgSO_4 \cdot 7H_2O$), 0.109 g P (KH_2PO_4) and 0.207 g K (KH_2PO_4 and K_2SO_4) per liter solution (Chen and Cutright, 2002). The treatment groups contained nutrient materials, synthetic acid mine drainage (SAMD) and different level of CA and rhizosphere bacteria. SAMD contained 0.40 g Fe ($FeSO_4 \cdot 7H_2O$), 0.03 Al g ($Al_2(SO_4)_3 \cdot 16H_2O$), 0.02 g Mn ($MnSO_4 \cdot H_2O$), 0.20 g Ca ($CaSO_4 \cdot 2H_2O$), 0.10 g Mg ($MgSO_4 \cdot 7H_2O$), 0.05 g Na ($Na_2SO_4$) per litter of distilled (DI) water, the pH of SAMD was adjusted to 3.5 with H_2SO_4 (Senko et al., 2008). The dosages of CA were selected based on previous research (Mihalík et al., 2010) and the concentrations of metals in solution.

There were nine different conditions: control solution without adding CA and rhizosphere bacteria (control); SAMD without CA and with or without rhizosphere bacteria (NCWB and NCNB); SAMD amended with low level of CA (0.021 g/l CA) and with or without inoculating with rhizosphere bacteria (LCWB and LCNB); SAMD added with middle level of CA (1.061 g/l CA) and with or without rhizosphere bacteria (MCWB and MCNB); SAMD with high level of CA (2.101 g/l CA) and with or without rhizosphere bacteria (HCWB and HCNB). Triplicate reeds were used for each experimental condition. Seedlings of reeds were cultured in the hydroponic solutions in glass gars (diameter 10 cm; volume 2000 ml) for 3 months and then harvested for analysis.

2.3. Rhizosphere bacteria: Isolation, enrichment and inoculation

The rhizosphere bacteria were collected from the root zone of reeds grown at the AMD site, as they had already acclimatized to the hostile environment. Five g of rhizosphere soil was transferred into the 100 ml nutrient solution that contained 1.848 g $(NH_4)_2SO_4$, 0.492 g $MgSO_4 \cdot 7H_2O$, 1.8 g glucose, 0.25 g trypticase soy broth per liter, with a pH of 3.5 (Johnson, 1995). Bacterial cells were separated from the soil by centrifugation (900 × *g*) for 15 min (IECentra-4B Centrifuge, USA). They were then enriched in growth medium which contained 1.848 g $(NH_4)_2SO_4$, 0.492 g $MgSO_4 \cdot 7H_2O$, 1.8 g glucose, 0.25 g trypticase soy broth, 0.40 g Fe ($FeSO_4 \cdot 7H_2O$), 0.03 g Al ($Al_2(SO_4)_3 \cdot 16H_2O$), 0.02 g Mn ($MnSO_4 \cdot H_2O$) in one liter DI water. The pH of the solution was adjusted to 3.5 by H_2SO_4.

After transferring into the hydroponic solution, parts of the reeds were inoculated with rhizosphere bacteria to assess the impacts of bacteria on metal plaque formation and metals uptake of plants. First, bacterial cells were separated from 10 ml growth medium by centrifugation at 900 × *g* for 15 min. Cells were re-suspended in 5 ml nutrient solution. Then the cell suspensions were injected into the hydroponic solution by sterile syringes.

2.4. Plaque extraction and plant digestion

Upon harvesting, the roots and rhizomes of reeds were gently washed by DI water, and then immersed in DI water overnight. Next, the remaining plants were further separated into roots, rhizomes, stems and leaves. The fresh roots or rhizomes were assigned to cold dithionite–citrate–bicarbonate (DCB) extraction techniques (Taylor and Crowder, 1983a) to determine the concentration of metal plaque on the surface of roots or rhizomes. After extraction, the rinsed roots or rhizomes were air dried as the other plant tissues. After drying, the tissues were milled and digested (Chen and Cutright, 2002; Ali et al., 2002): one g of plant tissues was soaked in 20 ml of nitric acid (70 percent) for 6 h. The mixture was heated and boiled to 10 ml. Then, 4 ml of perchloric acid (70 percent) was added and the mixed solution was refluxed at 150 °C for 90 min. Finally, the plants tissues were all digested into solution and the solution was diluted to 20 ml with DI water. The solution was kept in the refrigerator until analyzed by ICP-MS. Triplicate roots, rhizomes, stems or leaves from each reed were used for plaque extraction or plant digestion experiments. The extraction and digestion solution was kept in the refrigerator until analyzed by inductively coupled plasma mass spectrometry (Perkin-Elmer Plasma 400 Spectrophotometer ICP–MS).

2.5. Plate counts for bacteria

Solution was collected into pre-sterilized bottles during plant harvesting. As important groups of bacteria at AMD site, acidophilic heterotrophs in solution were enumerated on WAYE medium (Johnson, 1995), while iron oxidizing bacteria (Fe(II) OB) were cultured on FETSB medium (Senko et al., 2008). Bacteria were cultured for four weeks and the colony forming units (CFU) were counted.

2.6. Statistical analysis

The experiment was two-factor (CA and bacteria) completely randomized design with subsamplings (or two-stage nested design). The main factors were CA with four levels (without CA, low, middle and high level of CA) and rhizosphere bacteria with two levels (with or without inoculating rhizosphere bacteria). Data on numbers of rhizosphere bacteria, metal plaque formation and metal uptake in reeds were analyzed with general linear model using the Minitab statistical package (Minitab 16). Differences between specific CA amendments and rhizosphere bacteria inoculation were identified by Tukey's "HSD" procedure ($\alpha = 0.05$).

3. Results and discussion

3.1. Rhizosphere bacteria

As expected, the solution inoculated with rhizosphere bacteria possessed more Fe(II)OB and acidophilic heterotrophs than solution without adding rhizosphere bacteria throughout the entire period (Table 1). For example, the NCNB solution possessed $4.22 \pm 0.18 \times 10^3$ CFU/ml acidophilic heterotrophs and $3.80 \pm 0.20 \times 10^2$ CFU/ml Fe(II)OB, while the NCWB solution had $3.90 \pm 0.50 \times 10^5$ CFU/ml acidophilic heterotrophs and $1.92 \pm 0.10 \times 10^4$ CFU/ml Fe(II)OB.

It was not surprising that the numbers of heterotrophs were higher in solutions added with high level of CA than that without CA or with only a low level of CA. For instance, the LCWB solution possessed $5.40 \pm 0.20 \times 10^5$ CFU/ml acidophilic heterotrophs, while the HCWB solution had $1.27 \pm 0.09 \times 10^6$ CFU/ml acidophilic heterotrophs. In general, the more CA added, the more acidophilic heterotrophs were in the solution. This was due to the fact that CA can be used as carbon source by many microorganisms (Chen et al., 2006a). According to the results of Küsel et al. (2003), citrate can be consumed by rhizosphere bacteria at pH 3. Francis (1998) also pointed out that CA or metal-citrate complex can be metabolized intracellularly in bacteria by the enzymes.

Fe(II)OB was found in the rhizosphere of reeds under all the treatment conditions. Previous research also reported the presence of lithotrophic Fe(II)OB in the rhizosphere of wetland plants (Neubauer et al., 2007). Contrary to acidophilic heterotrophs, the

Table 1
Population of rhizosphere bacteria under different conditions.

Treatment	WAYE (CFU/ml)	FETSB (CFU/ml)
Control	$1.50 \pm 0.30 \times 10^3$ d	/
NCNB	$4.22 \pm 0.18 \times 10^3$ c	$3.80 \pm 0.20 \times 10^2$e
NCWB	$3.90 \pm 0.50 \times 10^5$ b	$1.92 \pm 0.02 \times 10^4$ a
LCNB	$6.70 \pm 0.70 \times 10^3$ c	$3.60 \pm 0.40 \times 10^2$ e
LCWB	$5.40 \pm 0.20 \times 10^5$ b	$1.56 \pm 0.06 \times 10^4$ b
MCNB	$1.30 \pm 0.10 \times 10^4$ c	$2.10 \pm 0.10 \times 10^2$ e
MCWB	$1.13 \pm 0.03 \times 10^6$ a	$9.10 \pm 0.50 \times 10^3$ c
HCNB	$3.23 \pm 0.37 \times 10^4$ c	$9.00 \pm 1.00 \times 10^1$ e
HCWB	$1.27 \pm 0.09 \times 10^6$ a	$2.76 \pm 0.16 \times 10^3$ d

/ Indicated blow detection limits; results were reported as average ± 1 standard deviation, $n = 10$. Different letters for each column indicated a significant difference at $p < 0.05$.