

# Changes in pH and organic acids in mucilage of *Eriophorum angustifolium* roots after exposure to elevated concentrations of toxic elements

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**Abstract** The presence of *Eriophorum angustifolium* in mine tailings of pyrite maintains a neutral pH, despite weathering, thus lowering the release of toxic elements into acid mine drainage water. We investigated if the presence of slightly elevated levels of free toxic elements triggers the plant rhizosphere to change the pH towards neutral by increasing organic acid contents. Plants were treated with a combination of As, Pb, Cu, Cd, and Zn at different concentrations in nutrient medium and in soil in a rhizobox-like system for 48–120 h. The pH and organic acids were detected in the mucilage dissolved from root surface, reflecting the rhizospheric solution. Also the pH of root–cell apoplasm was investigated. Both apoplasmic and mucilage pH increased and the concentrations of organic acids enhanced in the mucilage with slightly elevated levels of toxic elements. When organic acids concentration was high, also the pH was high. Thus, efflux of organic acids from the roots of *E. angustifolium* may induce rhizosphere basification.

**Keywords** *Eriophorum angustifolium* · Heavy metals and As · Organic acids · pH changes

## Introduction

Aquatic plants can tolerate high metal concentration in their immediate surroundings and this can be due to increased metal exposure which may have led to the development of metal tolerance (Otte et al. 2004). Roots of wetland and terrestrial plants may cause rhizosphere basification after heavy metal exposure (Blossfeld et al. 2010; Stoltz and Greger 2002; Zeng et al. 2008) as a way to tolerate elevated level of toxic elements. Blossfeld et al. (2010) reported rhizosphere basification by roots of alpine pennycress and ryegrass in metal contaminated soils as a permanent feature. In sulfidic mine tailings, two cotton grass species, *Eriophorum angustifolium* and *Eriophorum scheuchzeri*, maintained a pH of ~6 while in controls without plants, the pH was reduced to ~3 (Stoltz and Greger 2002). The root-induced basification by *E. angustifolium* and *E. scheuchzeri* was prominent on tailings having low buffering capacity and high contents of sulfides and elevated levels of toxic elements (Stoltz and Greger 2006). In hydroponics, rice (*Oryza sativa*) roots significantly increased the rhizosphere pH under Cr stress (Zeng et al. 2008). Imbalance of the absorption of cations and anions, CO<sub>2</sub> generated by rhizosphere respiration, a secretion of organic acids, H<sup>+</sup>, other chemical components from the roots, and root-associated micro-organisms are the main components establishing the rhizosphere pH (Curtin and Wen 2004; Song et al. 2004).

The rhizosphere pH changes that occur as a result of root activities influence the biogeochemistry of different elements (Hinsinger et al. 2006), depending on the element

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properties, which ultimately influence the element uptake. An increase in rhizosphere pH, by reducing the availability of toxic trace elements, would be a way to reduce the exposure of plants to potentially toxic trace metals. The pH increase by *E. angustifolium* and *E. scheuchzeri* roots limits the leakage of Cd, Cu, Pb, and Zn, but not the leakage of As from tailings (Stoltz and Greger 2002). Total copper concentration decreased threefold in the wheat rhizosphere solution due to root-induced basification (Bravin et al. 2009). This may be due to precipitation of metals at higher pH (VanLoon and Duffy 2000). On the contrary, a decrease in pH increases the soluble metal fraction of Cd and Zn, which significantly enhance metal uptake in *Thlaspi caerulescens* (Wang et al. 2006). The increased metal availability at low pH can cause severe injury and even death to trace-metal sensitive species or at least enhance the concentration of potentially toxic elements in edible plant organs.

After metal exposure, plant roots excrete different organic acids (Haoliang et al. 2007; Qin et al. 2007; Zeng et al. 2008). The released organic acids may protect the plant roots by limiting metal transport across the biological membranes due to metal-ion complexes with organic anions (Kochian et al. 2004). The organic acids may increase or decrease the rhizosphere pH depending on the form in which they are released. Organic acids are released as anions, and therefore, their release should be balanced by release of cations. Metal stress may decrease the efflux of protons by impairing the H<sup>+</sup>-ATPase pumping activities shown in some plants, e.g. *Cucumis sativus* (Janicka-Russak et al. 2008), sugar beet, and wheat (Lindberg and Wingstrand 1985; Lindberg et al. 2004). In this context, it is likely that metal exposure results in a defense mechanism releasing organic anions from the plant roots. The anions ultimately consume protons, particularly when the substrate pH is low. Thus, besides direct binding of organic anions with metals, the metal immobilization depends on the rhizosphere basification by plant roots.

The aim of the study was to further clarify the findings that roots of *E. angustifolium* increase pH, which was shown by Stoltz and Greger (2002). Stoltz and Greger (2006) also suggested that the roots of *E. angustifolium* could cause the rhizosphere pH increase when the available element concentration in the rhizosphere reaches a toxic, but not detrimental, level. Therefore, current studies were designed to investigate if slightly elevated levels of free toxic elements trigger a pH increase in the mucilage of *E. angustifolium* roots. Furthermore, if the pH change is related to a change in organic acid concentration of the mucilage, and if the root apoplasmic pH is changed accordingly, this should indicate an elemental toxicity. The results will give us a better understanding of the mechanism behind the phytostabilizing process, where *E. angustifolium* prevents heavy metal

release by keeping a neutral pH in mine tailings as described by Stoltz and Greger (2002).

## Material and methods

### Apoplasmic pH

Seeds of *E. angustifolium*, collected from the Boliden mining area in northern Sweden (64°52'N, 20°22'E) were grown in a green house (Temp 20±2 °C, RH~65 %), in small plastic pots carrying soil (K-fjord, Hasselfors Garden). After 6 weeks, seedlings were washed with distilled water, mounted in Styrofoam plates, and placed in a growth chamber in 1-L black containers (five to six plants per container) containing aerated 1 % Hoagland solution. The temperature in the chamber was 20±2 °C, RH 70 % with 16-h/8-h light/dark conditions. To acclimatize the plants, the concentration of the nutrient solution was gradually increased up to 25 % and replenish once a week.

After 6 weeks, plants were moved to 150-mL black containers with 25 % Hoagland solution (pH5.5). A combination of the elements Cd, Cu, Pb, Zn, and As (as CdCl<sub>2</sub>, CuCl<sub>2</sub>, PbCl<sub>2</sub>, ZnCl<sub>2</sub>, and NaAsO<sub>2</sub>) was used to mimic an elevated level of toxic elements present in mine tailings, e.g., that of pyrite from Kristineberg mine tailings impoundment in Sweden (Stoltz and Greger 2002). The elements were added to final concentrations 0, 15, 25, 35, and 50 μM of each element and cadmium was added to final concentrations of 0, 1.5, 2.5, 3.5, and 5.0 μM. The concentration ranges found in mine tailings of Kristineberg were; 0.06–4 μM Cd, 30–1,900 μM Zn, 0.3–50 μM Cu, 0.3–38 μM Pb, and 0.15–1.8 As (calculated from Stoltz and Greger 2002). Seedlings were exposed to the different treatments for 3 days using three replicates for each treatment.

For apoplasmic pH measurement, roots were washed with deionized water and placed in 5 μM Oregon Green 488-dextran (MW=10,000, Invitrogen) for 30 min. Thereafter, we followed the vacuum infiltration technique as described by Geilfus and Mühling (2011), in which roots were infiltrated with 5 μM Oregon Green into the root apoplasm. Subsequently, roots were washed again with distilled water to remove adhering dye and cut into pieces. Root segments were placed between an objective plate and coverslip and used to measure fluorescence emission intensity by an epi-fluorescence microscope (Axiovert 10; Zeiss Oberkochen, Germany). The excitation and emission wavelengths were 485/436 and 500–530 nm, respectively.

### Organic acids and pH in mucilage

In order to investigate pH and organic acid-concentration changes of root mucilage, *E. angustifolium* plants were grown

**Table 1** pH and organic acid concentration (milligram per gram of root dry weight) in root mucilage of *E. angustifolium* after 2-days exposure to a mixture of toxic elements at different concentrations in a rhizobox-like system.  $n=3$ , mean $\pm$ SE

Cu, Pb, Zn, As (ng/g)	Cd (ng/g)	pH	Organic acid concentration (mg g <sup>-1</sup> root dry weight)					
			Oxalic	Citric	Malic	Formic	Acetic	Succinic
0	0	7.11 $\pm$ 0.06c	7.18 $\pm$ 1.69b	0.94 $\pm$ 0.28a	2.03 $\pm$ 0.46a	0.63 $\pm$ 0.11c	0.66 $\pm$ 0.17a	0.41 $\pm$ 0.07b
15	1.5	7.68 $\pm$ 0.09a	3.75 $\pm$ 1.30b	2.24 $\pm$ 1.03a	2.38 $\pm$ 0.13a	0.73 $\pm$ 0.16c	0.92 $\pm$ 0.15a	1.20 $\pm$ 0.03b
25	2.5	7.86 $\pm$ 0.12a	17.8 $\pm$ 3.72a	3.45 $\pm$ 0.89a	2.97 $\pm$ 0.60a	4.19 $\pm$ 0.84a	1.69 $\pm$ 0.62a	3.17 $\pm$ 1.26a
35	3.5	7.16 $\pm$ 0.08b	1.82 $\pm$ 0.33b	3.88 $\pm$ 1.05a	1.60 $\pm$ 0.35a	1.28 $\pm$ 0.13b	0.88 $\pm$ 0.12a	1.81 $\pm$ 0.52b
50	5.0	7.27 $\pm$ 0.08b	2.62 $\pm$ 0.74b	1.85 $\pm$ 0.50a	2.17 $\pm$ 0.71a	1.27 $\pm$ 0.36b	0.90 $\pm$ 0.05a	1.03 $\pm$ 0.11b

Different letters (a–c) indicate significant difference ( $P\leq 0.05$ ) in organic acids concentrations and pH, respectively, at different element treatments using Tukey's HSD test.

in hydroponics for 12 weeks and transferred to a rhizobox-like system containing non-sterile humid soil (Greger 2005). A 25- $\mu$ m nylon net in the rhizoboxes prevented root penetration into the soil. The rhizobox soil (K-fjord, Hasselfors Garden, pH6.0) was spiked with CdCl<sub>2</sub>, CuCl<sub>2</sub>, PbCl<sub>2</sub>, AsNaO<sub>2</sub>, and ZnCl<sub>2</sub> six weeks in advance and concentrations added were 0, 15, 25, 35, and 50 ng/g soil (Cadmium concentration was 10 times lower than the other elements). After 48 h, plants were taken from the rhizoboxes, the roots were rinsed with 13 ml of redistilled water for 30 s. The solution was filtered through 0.45- $\mu$ m Millipore filter (Millex-HA, Millipore) and poured into 5-mL vessels (Greiner Bio One). For organic acids analysis, samples were mixed with NaOH (final concentration 0.01 M NaOH). The samples for oxalic acid and pH analysis were not treated with NaOH. The samples were immediately freeze-dried with liquid nitrogen and stored at  $-80^\circ\text{C}$  until analysis.

The freeze-dried samples were used for organic acids analysis after addition of 200  $\mu$ L redistilled water. Samples of 25  $\mu$ L were injected manually into an ion chromatograph and run for 30 min to measure conductivity. A Dionex ion chromatographic system was used, consisting of a 4,500 gradient pump, an AMMS-ICE II anion ion-exclusion micro-membrane suppressor, an ED50A electrochemical detector, a DS3-1 conductivity cell D23 detection stabilizer, and an IonPac ICE-AS6 analytical column (Dionex Corporation, Sun Valley, CA, USA). The samples were eluted isocratically with 0.4 mM heptafluoro butyric acid, and the flow rate of the eluent was 0.8 mL/min. The reagent was 5 mM tetrabutyl ammonium hydroxide, flowing at a rate of 5 mL/min. The organic acids exudates were identified using standard solutions of organic acids. Formic acid (2.4 g/L), citric acid (2.5 g/L), succinic acid (2.5 g/L), acetic acid (2.5 g/L), and malic acid (2.5 g/L) were also added to the samples to allow estimation of the exudate concentrations with a detection limit of approximately 0.1 mg/L.

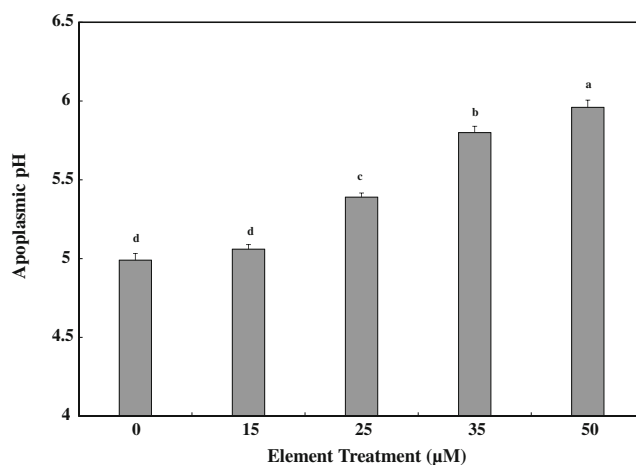
Oxalic acid was analyzed by a method modified from Mayer et al. (1979). Oxalate was precipitated by adding

1 mL of 0.1 M CaCl<sub>2</sub> to 1 mL of sample. The mixture was centrifuged at 13,000 $\times g$  for 40 min. The supernatant was removed with a suction pump and the pellet was dissolved in 1 mL of 25 mM H<sub>2</sub>SO<sub>4</sub>. During oxalic acid analysis with Dionex ion chromatographic system, 0.1 mM H<sub>2</sub>SO<sub>4</sub> was used at a flow rate of 0.8 mL min<sup>-1</sup>. Standard addition (0.63 g/L) was used and the recovery rate was at least 95 %.

The pH of root exudates was detected in freeze-dried samples dissolved in 200  $\mu$ L of redistilled water using a pH meter, Metrohm 744, with a combined LL micro-pH glass electrode.

#### Statistics

Statistical analysis of data was performed with simple regression and analysis of variance by using a statistical program "R" (version 2.13.0). Before statistical analysis, data was normalized by proper transformations when necessary.



**Fig. 1** Apoplasmic pH of *E. angustifolium* roots after exposure of plant roots to a combination of Cu, Pb, Zn, As (0, 15, 25, 35, and 50  $\mu\text{M}$ , indicated in figure) and Cd (0, 1.5, 2.5, 3.5, and 5.0  $\mu\text{M}$ ) in hydroponics for 3 days. Different letters indicate significant difference ( $P\leq 0.05$ ).  $n=3$ , mean $\pm$ SE

Tukey's honest significant difference test (HSD test) was used for detection of differences between the treatments, significant levels at  $p \leq 0.05$ .

## Results and discussion

In all treatments, the pH of the mucilage was higher in the presence of toxic elements compared with the control. The pH increase culminated at the two lowest toxic element treatments (Table 1). The root-induced basification after the uptake of toxic ions may be due to cation/anion imbalance, release of  $\text{OH}^-$  or  $\text{HCO}_3^-$  or  $\text{CO}_3^{2-}$ , or release of organic anions. The results of present study corroborate the finding of Zeng et al. (2008) who found a pH increase in the rhizosphere of rice (*O. sativa*) with increasing chromium levels in the medium. The pH-modulation response was slightly diminished at very high treatment levels (Table 1). The involvement of roots in the pH change can be seen by the increase of apoplasmic pH by roots of *E. angustifolium* after exposure to the toxic elements (Fig. 1). The highest pH increase of 0.97 units was observed in the apoplasm of *E. angustifolium* roots at the highest treatment concentration. *E. angustifolium* is mostly confined to acidic soils (pH < 5) but it can occur in soils with a pH of 8 (Grime et al. 1988). Therefore, the pH increase in mucilage and apoplasm (Table 1, Fig. 1) in this case is probably due to the exposure of the toxic elements, which can inhibit the plasma membrane proton pump, thereby causing apoplasmic basification. Since the root apoplasm has a relative small volume compared with the mucilage, this inhibition is not the single reason for the pH increase of the mucilage.

Exposure of *E. angustifolium* roots to 25 ng/g Pb, Cu, Zn, As, and 2.5 ng/g Cd significantly induced higher exudation of formic, succinic, and oxalic acids, respectively, as compared to the other treatments (Table 1). The exudation of acetic, citric, and malic acids was not significantly influenced in all treatment. However, only small correlation exist between pH increase by exudates and the amount of organic acids after element exposure (data not shown). The pH increase of root exudates observed in *E. angustifolium* after 48 h of element exposure is, therefore, not fully dependent on the release of these acids. Besides organic acids, other organic molecules may be involved, which then are responsible for the raise of pH, e.g. polypeptides, called "rapid alkalization factors", as reported for poplar and tomato (Haruta and Constabel 2003; Pearce et al. 2001; Wheeler and Irving 2010).

Metal stress stimulated the increase of organic anions in the rhizosphere by acting through different existing mechanisms, which remained constant with time (Zhao et al. 2003) and/or by the activation of genes (Ma et al. 2000). The former reaction is likely to work in *E. angustifolium*, as its roots secrete citric, malic, and acetic acids and metal exposure further activates the

secretion of oxalic, formic, and succinic acids. This idea is consistent with the findings of Stoltz and Greger (2002), who reported that this species maintain a high pH on acidic tailings for long time.

The pH increase by organic acids may depend on co-release of organic anions with nutrient cations, e.g.,  $\text{K}^+$  (Qin et al. 2007). The proton-consuming capability of different organic anions depends on the number of carboxylic groups that they carry. The acids, which increased consisted of two (oxalic and succinic acids) and one (formic acid) carboxylic groups. Under non-sterile conditions, rhizosphere microbes may also alter the chemical composition of roots exudates, which can contribute to rhizosphere basification. For example, fungi are known to cause rapid basification of the extracellular medium (Vylkova et al. 2011). Therefore, the observed rhizosphere pH rise by *E. angustifolium* roots subjected to toxic elements can be a combined effect of element toxicity and microbial activity.

## Conclusion

Roots of *E. angustifolium* respond to moderate elevated levels of toxic elements by releasing different organic acids which temporarily increase the rhizosphere pH. At high level of toxic elements, the organic acids exudation, and pH rise responses are diminished probably due to elemental toxicity. Our results suggested that *E. angustifolium* is a suitable plant for remediation of acidic metal polluted soils, as it can keep a high rhizosphere pH. This mechanism in turn enhances metal binding to the substrate, leading to enhanced phytostabilization of toxic elements. The present results focus on short-time effects of heavy metals because long-term investigations concerning plant survival and function were shown earlier by Stoltz and Greger (2002, 2006).

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