Different patterns of organic acid exudation in metallophyte and agricultural plants at increasing copper levels

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Abstract
Copper (Cu) accumulation in shoot and root tissues and low molecular weight organic acids (LMWOA) root exudation have been studied in two metallophytes from Central Chile (*Oenothera affinis* and *Imperata condensata*) and two agricultural plants (*Lupinus albus* and *Helianthus annus*) growing under hydroponics conditions at increasing Cu levels (0, 0.125, 0.250, 0.5, 1 and 2 mg Cu/L). The plants were grown in mineral solution for four weeks and after that in Cu-added solution for ten days, harvested and analyzed after this time. All the plant species showed high Cu accumulation, highlighting *O. affinis* with 116 and 2657 mg Cu/kg in shoots and roots, respectively. Strong differences in the LMWOA exudated by the different plant species were determined. *O. affinis* exudated high amounts of succinic acid, while *I. condensata* exuded citric and oxalic acids. Acid root exudations from agricultural plants were mainly composed by citric acid. This result suggests that LMWOA root exudates by metallophytes play an important role against high Cu levels in polluted soils, being an important factor for plant selection in the design and implementation of phytoremediation programs.

Key Words
Cu tolerance, metallophytes, organic acid exudation, phytoremediation.

Introduction
The main sources of metals in the environment are anthropogenic, such as agriculture, domestic and industrial wastes and mining, being the latter the more important (Ginocchio *et al.* 2004). Under this environmental stress, some plants have developed diverse mechanisms for tolerating high metal levels such as exudation of low molecular weight organic acids (LMWOA) (Tao *et al*. 2004). These exudates play an important function mobilizing low mobile/soluble nutrients (e.g., P, Fe, Zn), increasing microbial activity and complexing metals thus affecting the metal bioavailability. In this sense, the extracellular metal complexation could be an important adaptative mechanism for plant establishment in nutrient deficient soils or in those polluted with high metal levels (Shen *et al.* 2002).

On the other hand, it has been found that LMWOA are involved in the metal transport and storage in plants (Nigam *et al.* 2001). Under high Cu levels the exudation of LMWOA by agricultural plants has been widely studied, and several reports have shown that roots might exude diverse LMWOA, including citric, oxalic and succinic acid, which could play a important role in alleviating Cu toxicity (Quartacci *et al.* 2009). However, the exudation of LMWOA by plants tolerant to high Cu levels has not been extensively studied. In this way, there are no studies which contrast the Cu tolerance, Cu accumulation and exudation of LMWOA between Cu metallophyte and agricultural plants. Based on the aforementioned we hypothesized that LMWOA exudation may be a crucial Cu tolerance mechanism developed by metallophytes naturally growing in Cu polluted areas.

Therefore, the aim of this work was to determine the exudation pattern of LMWOA in mineral solutions, and Cu concentration of two metallophyte species and to contrast their behavior with two agricultural plants growing in the same conditions, for analyzing its possible use in phytoremediation.

Methods
Biological material and culture conditions
Commercial seeds of the agricultural plant species *Helianthus annus* and *Lupinus albus* cv. Rumbo-B, and seeds of *Oenothera affinis* obtained *in situ* in Cu polluted soils were sown. Additionally, stolons of *Imperata condensata* were collected from the polluted area to produce plantlets. The collect area was a Mediterranean ecosystem strongly affected by the deposit of metal-rich particles, located approximately at 1.5 km southeast from the Ventanas smelter (CODELCO), in the Puchuncaví valley, Central Chile (32°46′ 30″ S 71° 28′ 17″

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W). All seedlings were grown in perlite/sand/vermiculite substrate (1:1:1, v:v:v), supplemented with sterile ddH_2O and maintained in a growth chamber until roots reached > 2 cm-length. Then, the plants were transferred to 1-L polyethylene containers and were continuously aerated by air pumping. The nutrient solution used was 1.3 m*M* $MgSO_4$, 2.0 m*M* $Ca(NO_3)_2$, 2.0 m*M* KNO_3, 2.0 m*M* K_2HPO_4, and (in µ*M*) 0.2 µ*M* $CuSO_4$, 1.0 µ*M* $ZnSO_4$, 2.0 µ*M* $MnCl_2$, 20 µ*M* H_3BO_3, 0.1 µ*M* $(NH_4)_6Mo_7O_{24}$, and 200 µ*M* FeEDTA. The containers were placed into a plant growth chamber room under controlled conditions. After 4 weeks growing in this culture conditions the nutrient solution was replaced by the same solution described above, but supplied with 0, 0.125, 0.5, 1.0 and 2.0 mg Cu/L as $CuSO_4$. This test solution was replaced every two days to keep constant concentrations of nutrients and Cu. The plants grew in these conditions for 10 days, and after the root exudates were collected using the methodology proposed by Rosas *et al.* (2007) with minor modifications.

Measurements
Plants with intact roots were rinsed thoroughly with 50 mL of deionized water (≤ 1 µS/cm) and then were immersed in deionized water under constant aeration for 1 h. The solution was filtered (0.22 µm) and freeze-dried. In order to quantify the concentration of LMWOA the residue was resuspended in 300-500 µL of deionized-sterilised water for HPLC injection. The separation was conducted on a 250 × 4 mm reverse phase column (LiChrospher 100 RP-18 5 mm particle size; Merck, Darmstadt, Germany). Sample solutions (20 µL) were injected onto the column and 200 m*M* orthophosphoric acid (pH 2.1) was used for isocratic elution, with a flow rate of 1 mL/min and UV detection at 210 nm. Identification of organic acids was performed by comparison of retention times and absorption spectra with standards for each organic acid. After to obtain the root exudates, the plants were cut separating shoot and roots, washed with distilled water, oven-dried at 60 ºC for 48 h and weighed. The tissue samples obtained were crushed and converted into ash in a furnace and digested using a $H_2O/HCl/HNO_3$ mixture (8/1/1 v/v/v). The Cu concentrations were determined by Atomic Absorption Spectroscopy (Perkin-Elmer 3110).

Results
Copper accumulation
All the plant species studied showed Cu concentrations above normal (superior to 20 µg Cu/g DW, Adriano 2001), presenting values between 14 and 40 µg Cu/g DW in shoots, with the exception of *O. affinis* which presented a mean accumulation of 116 µg Cu/g DW at the highest Cu concentration (Figure 1, A). However, the highest differences in Cu concentration were registered in the root, where all species studied showed a sharp increase directly related with the increase in Cu concentration in the solution (Figure 1, B), reaching means of 330-660 µg Cu/g DW in the roots of all species at the highest Cu level, with the exception of *O. affinis* which at the same conditions sowed up to 2660 µg Cu/g (4 times higher than concentration in shoots and roots of the other species evaluated) (Figure 1).

Root exudates
Four different LMWOA were detected: succinic acid, oxalic acid, citric acid and small amounts of fumaric acid, which were strictly dependent of the plant species analyzed and the amount of Cu added to the solution (Figure 2). Succinic acid was principally exudated by the metallophyte *O. affinis,* which exuded high amounts at increasing Cu concentration, reaching values of 1048,56 µmol/h DW at the higher Cu level (Figure 2C). On the other hand, *Imperata condensata* exhibited LMWOA exudation rate was very variable under different Cu conditions. At low Cu concentrations (between control treatment and 0.125 mg Cu/L) *I. condensata* showed a low exudation of succinic acid (Figure 2C); however, at increasing Cu concentrations this metallophyte produced large amounts of citric and oxalic acid, reaching values of 164,05 and 1,61 µmol/ h.g DW respectively at the highest Cu concentrations (Figure 2 A, B). Root exudates of *L. albus* and *H. annus* contained exclusively citric acid (Figure 1A), which was exuded in high amounts, reaching on an average of 46,01 and 51,44 mol/h.g DW; however, the concentration of citric acid remained relatively constant with increasing copper concentrations in both plants.

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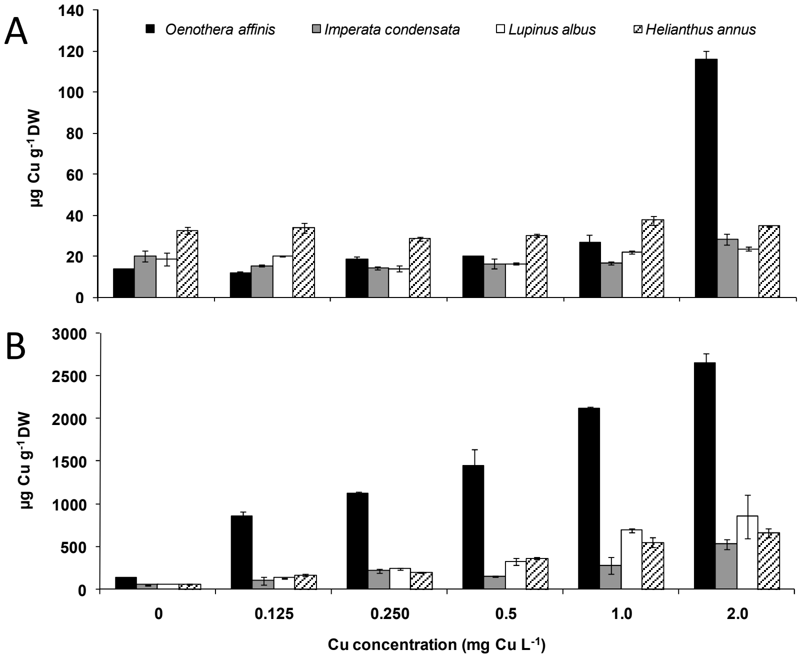

Figure 1. Copper (Cu) concentration in (A) shoots and (B) roots of four plants species in response to increasing Cu levels (0; 0.12; 0.250, 0.5; 1.0 and 2.0 mg Cu/L). Bars denote means ± S.E (n = 6). DW= Dry weight.

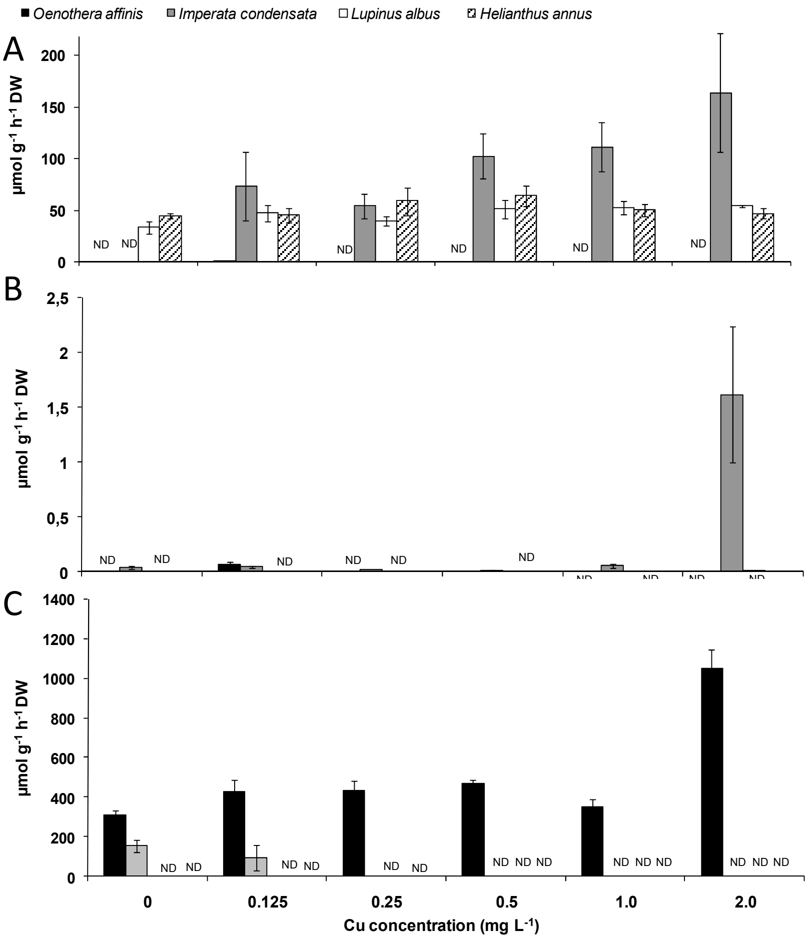

Figure 2. Exudation of low molecular weight organic acids in four plant species in response to increasing copper levels (0; 0.12; 0.250, 0.5; 1.0 and 2.0 mg Cu/kg). A) Citric acid, B) oxalic acid, C) succinic acid. Bars denote means ± S.E (n = 6). DW= Dry weight, ND= Not detected.

Conclusion

These results suggest that the different metallophytes and agricultural plants present differences with respect to its Cu tolerance. If metal tolerance is considered to be manifested as a series of physiological traits (including root elongation, metal uptake and accumulation; Baker and Walker 1990), the most tolerant species would be the metallophyte *O. affinis*, due to its high Cu accumulation capacity. Similarly, the differential exudations of LMWOA by plants, and the large amounts exuded by the metallophytes suggest that this is an important defense mechanism developed to tolerate high Cu concentrations. Further studies aimed to investigate the metal binding properties of each compounds released by roots and the possible involvement of other exudates (phenols) are necessary in order to assess their role in Cu tolerance and accumulating capacity of the metallophytes and agricultural plants as technological tool to be used in potential phytoremediation programs in Cu polluted soils.

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