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Effect of heavy metals and organic matter on root exudates (low molecular weight organic acids) of herbaceous species: An assessment in sand and soil conditions under different levels of contamination☆

M.M. Montiel-Rozas*, E. Madejón, P. Madejón

Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS-CSIC), Av. Reina Mercedes 10, PO Box 1052, 41080, Sevilla, Spain

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ABSTRACT

Bioavailability of heavy metals can be modified by different root exudates. Among them, low molecular weight organic acids (LMWOAs) play an important role in this process. Three plant species (*Poa annua*, *Medicago polymorpha* and *Malva sylvestris*), potentially used for phytoremediation, have been assessed for both metal uptake and LMWOAs excretion in contaminated environments with different concentrations of Cd, Cu and Zn. The experiments have been carried out in washed sand and in three contaminated soils where two organic amendments were added (biosolid compost and alperujo compost). The most abundant LMWOAs excreted by all studied plants were oxalic and malic acids, although citric and fumaric acids were also detected. The general tendency was that plants responded to an increase of heavy metal stress releasing higher amounts of LMWOAs. This is an efficient exclusion mechanism reducing the metal uptake and allowing the plant growth at high levels of contamination. In the experiment using wash sand as substrate, the organic acids composition and quantity depended mainly on plant species and metal contamination. *M. polymorpha* was the species that released the highest concentrations of LMWOAs, both in sand and in soils with no amendment addition, whereas a decrease of these acids was observed with the addition of amendments. Our results established a clear effect of organic matter on the composition and total amount of LMWOAs released. The increase of organic matter and nutrients, through amendments, improved the soil quality reducing phytotoxicity. As a result, organic acids exudates decreased and were solely composed of oxalic acid (except for *M. polymorpha*). The release of LMWOAs has proved to be an important mechanism against heavy metal stress, unique to each species and modifiable by means of organic amendment addition.

1. Introduction

Heavy metal bioavailability is the most important factor to be monitored in the restoration process of a contaminated soil. Such bioavailability depends on several factors as soil characteristics and plant species growing in this soil. An unclear idea exists regarding the effect of rhizospheric processes on heavy metal availability (Kidd et al., 2009). Root exudates both high-molecular weight (polysaccharides and proteins) and low-molecular weight (i.e. amino acids, organic acids, sugars, phenolics) compounds play an important role in these rhizospheric processes (Bais et al., 2006). Among them, low-molecular weight organic acids (LMWOA) are the most abundant and reactive with metals (Koo et al., 2010).

The changes in the rhizosphere produced by root exudates vary according to the plant species growing in each soil. In addition, the exudation of organic acids, both in quantity and in relative proportions, is directly affected by the presence of metals in the soil (Meier et al., 2012). These key aspects should be considered in phytostabilisation since the choice of the plant species generates a variation at rhizosphere level that results in an increase/decrease in the availability of metals and therefore determine the success of the stabilization strategy.

Understanding the role of organic acids in the plant tolerance to heavy metals is crucial for the successful implementation of phytoremediation technologies. Several previous studies have reported that the organic acids behave as natural chelating agent (Kim et al., 2010; Agnello et al., 2014) and can involve a pH decrease leading to

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* Corresponding author.
E-mail address: mmontielr@irnas.csic.es (M.M. Montiel-Rozas).

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the acidification of the rhizosphere (Zhixin et al., 2013; Seshadri et al., 2015). Apart from the scarce and unclear knowledge of the role of organic acids, metal uptake and accumulation in plants is a complex process and the physiological mechanisms involved are still greatly unknown. Metal plant response is complex, varying considerably between species, specific for different metals, and metal concentration-dependent (Arnetoli et al., 2008).

The experimental growth conditions (most studies have been conducted in hydroponics i.e. Zhao et al., 2001; Meier et al., 2012; Hawrylak-Nowak et al., 2015) affect to the development and size of the root, and therefore can affect the excretion of organic acids. Plants growing on artificial matrix (sand culture system) with the addition of contaminants through nutritive solution can help to understand plant uptake behaviour in a pollution gradient. Even more, this type of experiments allows a more accurate study of the roots, and their exudates, because their analyses are easy to handle, avoiding interferences due to soil particles (Liao et al., 2003). However, these types of studies need complementary experiments using soils from real contaminated areas as a matrix for plant growth. The interpretation of both experiments offers a wider knowledge about plant mechanisms of heavy metals uptake and accumulation and the response, at rhizosphere level, to stressful conditions created by contamination.

In the restoration of contaminated soils, the use of organic amendments is widespread (Ciadamidaro et al., 2015; Hattab et al., 2015; Montiel-Rozas et al., 2015) but the effect of them on LMWOA exudate by roots has been scarcely studied. A few previous studies have reported the increase of LMWOAs release in soil solution due to the amendment addition (Peña et al., 2015). Thus, it would interesting to evaluate the direct and indirect effects of the organic amendments on root exudates (concretely LMWOA), both in quantity and composition. The aims of the present study were: a) to test the response (in terms of LMWOA release) of three potential species to use in phytoremediation strategies to different Cd, Cu and Zn concentrations; b) to analyse the effect of the addition of organic amendments used in soil restoration (*alperujo* compost and biosolid compost) in the quantity and variety of LMWOAs released and c) to evaluate the effect of LMWOAs in the metal uptake of the aerial parts of the plants. For this purpose, we studied plant behaviour growing in artificial matrix (sand) and under soil conditions by using three contaminated soils differing in metal availability and organic matter content.

2. Material and methods

2.1. Experimental design

To assess the response of different plant families, the following species were used: *Poa annua* L. (Poaceae; PO), *Medicago polymorpha* L. (Leguminosae; ME) and *Malva sylvestris* L. (Malvaceae; MA). In case of *M. sylvestris,* a germination pre-treatment was applied to seeds (10 min at 80 °C; 24 h in distilled water).

Two microcosms experiments were carried out (1 and 2). The Experiment 1 ("washed sand and increasing gradient of contamination") was carried out in pots filled with washed sand in a greenhouse with a temperature of 23 ± 2 °C and increasing doses of metals solution (containing Cu, Cd and Zn) were added. Five treatments according to different contamination levels were established: Dose 1 (D1; 0.5 mg Cd/L, 5 mg Cu/L, 20 mg Zn/L); Dose 2 (D2; 1.5 mg Cd/L, 15 mg Cu/L, 60 mg Zn/L); Dose 3 (D3; 3 mg Cd/L, 30 mg Cu/L, 120 mg Zn/L); Dose 4 (D4; 6 mg Cd/L, 60 mg Cu/L, 240 mg Zn/L) and Dose 5 (D5; 10 mg Cd/L, 100 mg Cu/L, 400 mg Zn/L). Five replicates per doses and plant species were set up (75 pots). Contaminants solutions were prepared from $CdCl_2$, $CuSO_45H_2O$ and $ZnSO_47H_2O$ salts. To maintain the correct plant development, pots were irrigated with nutritive solution (Hoagland) every 3–4 days. After germination of seeds (1 cm of radicle emerged), contamination solutions were applied progressively for 3 weeks: 20 ml, 40 ml and 60 ml per pot respectively. One week after last contamination, it proceeded to organic acids extraction.

The Experiment 2 ("Metal contaminated soil") was carried outdoors in pots that were filled with three contaminated soils: Soil A and B from an area affected by a mine spill (Grimalt et al., 1999) and Soil C from an area chronically contaminated by metals (Tharsis mining area, Huelva). Total Cd, Cu and Zn in the soil were 1.70, 113 and 508 mg kg^{-1} respectively in soil A, 0.28, 88 and 121 mg kg^{-1} respectively in soil B and 0.65, 105 and 456 mg kg^{-1} in soil C.

In each soil and for each species three treatments with four replicates per treatment were established (108 pots). Treatments were: biosolid compost amended soil (BC), *alperujo* compost amended soil (AC) and non-amended soil (CO). Biosolid compost was collected from the composting plant "EMASESA" (Seville, Spain) and was produced by the mixture of sewage sludge and pruning from parks and gardens from Seville city. The *alperujo* compost (a semisolid by-product obtained from the two-phase centrifugation system for olive oil extraction) was prepared by the cooperative "Coto Bajo" Guadalcázar (Córdoba, Spain) by mixing alperujo with legume residues and manure from organic farming. The main characteristics of the amendments are reported in Ciadamidaro et al. (2015). A single addition of amendments (25 g per kg of soil) was made and afterwards (one week) seeds of the species were established.

The experiment was conducted for 6 months. The pots were regularly irrigated by dripping (three days per week) to ensure the plants water demand. Biomass harvesting was performed at the end of the experiment. In both experiments, containers were arranged according to a complete randomised block design.

2.2. Organic acids extraction and analysis

To measure the LMWOA release from roots in both experiments, complete plants were extracted carefully from of each pot and roots were carefully washed with distilled water. Each plant was placed in a tube and the complete root system of the plant was submerged in a 0.01 M $CaSO_4$ $2H_2O$ solution for 2 h under the same controlled climate conditions described for plant growth (Aulakh et al., 2001). The tubes were covered with aluminium foil to create dark conditions for roots. The extracts of root exudates were filtered to eliminate cell debris (0.45 μm) and kept at −20 °C until HPLC analysis. Finally, each root from the tubes was weighted (fresh and dry) for subsequent calculations.

Chromatographic analysis was conducted in an HPLC system (Waters 1525-Milford, MA) connected to an autoinjector 717 and a photodiodes detector (PDA) 2996. Chromatographic analysis was conducted on a reverse phase column (Synergi™ 4 μm Hydro-RP (250 × 4.6 mm), Phenomenex). The mobile phase was KH_2PO_4 buffered with 20 mM at pH 2.5. The injection volume was 25 μl and the wavelength was 220 nm.

The calibration line was obtained by external standards at a concentration range of 10–40 mg L^{-1} from which the quantification of the samples was performed with a correlation coefficient (R2) of 0.99 for each of the organic acids analysed. The detection limit was 0.05 mg/L for all organic acids. Identification of LMWOAs was performed by comparison of retention times and by addition of standards for each organic acid. The different retention times were 3.7, 5.8, 11.4, 12 and 14 min for oxalic, malic, citric and fumaric acids, respectively.