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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

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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.

**Keywords:** Rhizosphere, Phytoremediation**,** Amendments**,** Oxalic acid, Malic acid, Citric acid

## Capsule abstract.

Root excretion of LMWOAs has proved to be an important mechanism in response to heavy metal stress, which depends on plant species, level of contamination and soil organic matter.

## 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 the acidification of the rhizosphere (Zhixing 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 aim of the present study was: 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 80ºC; 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±2ºC 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 0.01M $CaSO_4$ $2H_2O$ solution for 2h 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 (0.45µm) and kept at -20ºC 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 x 4.6 mm), Phenomenex). The mobile phase was $KH_2PO_4$ buffered with 20mM 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 to 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 minutes for oxalic, malic, citric and fumaric acids, respectively.

**2.3. Soil and plant analysis**

Plant biomass of each pot was harvested at the end of both experiments, and fresh and dry weight was recorded. Vegetal material was washed with a 0.1N HCl solution and with distilled water. They were oven dried at 70 ºC and, finally, grounded and passed through a 500-µm stainless-steel sieve. Dried plant samples were digested by wet oxidation with concentrated $HNO_3$ under pressure in a microwave oven. Determination of Cd, Cu and Zn in the extracts was performed by ICP-OES (inductively coupled plasma-optical emission spectrometry). The accuracy of the analytical methods was assessed through three plant reference samples (INCT-TL-1, Tea leaves, INCT-OBTL-5 and NCS DC 73348).

Soil sampling was performed at the beginning and at the end of the Experiment 2, although only final data have been presented in this study. Soil pH was measured according to Hesse (1971). Pseudo-total concentrations of Cd, Cu and Zn in soil samples were determined by digestion with *aqua regia* in a microwave oven and the available concentrations of these elements were determined as described Houba et al. (2000). Cadmium, Cu and Zn in the extracts were determined by ICP-OES. Total organic carbon (TOC) was determined according to Walkley and Black (1934).

## 2.4. Statistical analysis

To test significant differences between studied variables one-way ANOVA was performed. It was carried out to analyse differences between species (considered as independent variable) in LMWOAs concentrations for each dose and soil-treatment combination, differences between heavy metal uptake and biomass for each dose (Experiment 1) and differences between pH and TOC in each soil-treatment combination. Normality and homoscedasticity of data were tested with Kolmogorov-Smirnov and Levene test, respectively. Non-normal variables were transformed prior to ANOVA by logarithmic transformation. Post-hoc analyses were based on Tukey's test when variances were equal whereas Dunnett's T3 test was used in case of unequal variances. Significance level used was 0.05.

A Spearman's correlation analysis was performed to determine the relation between LMWOAs, metal concentration in plant tissues and biomass (in case of Experiment 1) and between TOC, metal availability and LMWOAs (Experiment 2).

For Experiment 1, the Transfer Factor (TF; metal concentration in shoots divided by metal concentration in roots) was calculated for each species and dose.

To show clearly the trends found in the organic acid exudation under different conditions evaluated (soil-treatment-plant), it was performed a heatmap using *heatmap* function (stats package in R). A heatmap is a graphical representation of data where the similar values contained in a matrix are placed near each other according to the clustering. The similarity level is represented by a colour gradient. In addition, a dendrogram was added to the left side and to the top of the graphics. A Factorial Analysis was carried out to explain the interdependence between all variables through the factors obtained. For the experiment 1, the variables used were as follows: Plant biomass, LMWOAs, Cd, Cu and Zn contents in roots and shoots. For the experiment 2, TOC in soil, LMWOAs and Cd, Cu and Zn contents in shoot were used. In this case, the Factorial Analysis was performed for each treatment. The sampling adequacy was verified by KMO index (>0.6 in all cases). To maintain the independence of factors, Varimax rotation (orthogonal) was chosen. The extraction method was the Principal Component Analysis and, after establishing the rotated factors, the values taken by the factors on each observation were calculated by regression method.

## 3. Results

### 3.1. Experiment 1: washed sand and increasing gradient of contamination

#### *3.1.1. Biomass, metal accumulation and distribution in plant tissues*

Values of biomass yield did not differ significantly between the first three doses in ME and PO species (Fig. 1.A). As the metal concentration increased (doses 4 and 5) plant biomass decreased significantly, especially in case of ME. No differences were found between dose 4 and 5 for any of the species. In general, the highest values of biomass were found for PO, whereas the lowest were obtained for MA. By comparing biomass values between species at each dose, only significant differences were found at the highest doses of metals (4 and 5).

The three species maintained similar metal concentration in both roots and shoots at the lowest doses (1-3). At these contamination levels, the highest concentrations of metals were found in MA species for the three elements, except for Cu in shoots (Fig. 1.B).

Cadmium and Zn presented similar behaviour in plants; the accumulation in shoots was increasing slowly with the increment of the concentrations to doses 3 (showing significant differences between doses; Fig. 1.B). At highest doses (4 and 5), MA was the species with the highest Cd and Zn accumulation in aerial parts.

The pattern of metal accumulation in roots was similar to shoots (Fig. 1.B). Metal accumulation in roots was higher than in the aerial plant part with the exception of the Zn, which resulted in general higher values of Transfer Factor (TF) for this element, especially for MA plants (2.3 and 1.9 at doses 1 and 2, respectively). As the doses of the Zn in the media increased, TF values of PO and MA tended to decreased, whereas no clear tendency was detected for ME. TF values for Cd and Cu were <1 for all species tested.

*3.1.2. Low molecular weight organic acids*

In the experiment 1, the LMWOAs excretions from the roots varied among species (Table S1). In general, the highest concentrations of LMWOAs were found on the exudates of ME, whereas the lowest were obtained in MA. Despite the differences in LMWOA concentrations, ME and MA species showed a similar pattern along the contamination gradient from dose 3 (Fig. 2). From this contamination level, oxalic was the most abundant acid in the rhizosphere. Malic and fumaric acids were maintained at similar concentrations in lower doses whereas citric acid concentration increased progressively. In the case of PO, the pattern was completely different. Oxalic and malic acids showed a similar behaviour; a decrease from dose 1 to 2 was reported, followed by a slight increase at dose 3 and a subsequent increment at dose 4. At the highest dose, the main acids released by PO were oxalic and citric.

Apart from the fact that the highest levels of oxalic acid were measured in ME roots, significant differences with the other species were only found from dose 3. In the case of malic acid, significant differences were found at low doses (1 and 2), whereas from dose 3 results showed that exudates concentration did not differ between species. Citric acid released differed at all contamination levels except at dose 2. Fumaric acid was measured in all doses and species exudates. Although this organic acid was released at lowest amounts, the only differences were found at low Cd, Cu and Zn concentrations (dose 2).

*3.1.3. Factorial analysis and correlations.*

The main factor (Fact 1; explain 52.7% of variance) corresponds to the "Contamination" factor (Fig. 3). This factor was explained by an inverse relation between biomass and metal concentrations (both in root and shoot). Across the x-axis, factor scores were arranged according to an increase of metal concentration in rhizosphere according with higher doses in the media. The second factor ("Plant species") explained 22.4% of variance and it was related to LMWOAs. The analysis showed that the root exudates were mainly dependent on the plant species. The effect of different plant species on root exudates pattern was clearer in the case of MA since it was strongly negatively correlated with LMWOAs ($p<0.01$), particularly in low doses. At the highest dose the response of the MA was mainly described by factor 1.

In general, a significant inverse correlation between biomass and metal concentration in vegetal tissues was found at $p<0.01$ for all species, corroborating the results extracted from Factorial Analysis (Fact 1). However, correlations between heavy metals and LMWOAs varied according to the species. A positive correlation between Cu content in shoots of PO and citric and fumaric acids was found ($p<0.05$), whereas the increase of metals in roots was negatively correlated with malic acid ($p<0.05$). The highest concentrations of metals in roots and shoots of ME were accompanied by an increase of citric acid and a decrease of fumaric acid ($p<0.05$). Similarly, to PO, a negative correlation between malic acid and metals in roots (significant for Cd and Zn) was observed. By contrast, all metals both in roots and shoots (except Cu in root) of MA were significantly correlated with all LMWOAs: positively with oxalic, citric ($p<0.01$) and fumaric acid ($p<0.05$) and negatively with malic acid ($p<0.01$). Likewise, correlations found between different organic acids differed for each species. Positive correlations were found between oxalic-malic acids and citric-fumaric acids in case of PO and malic-fumaric acids in ME. However, all LMWOAs were correlated in case of MA (oxalic, citric and fumaric were correlated positively among them and negatively with malic acid).

## 3.2. Experiment 2: Metal contaminated soil

### *3.2.1. Soils*

Values of pH of the three studied soils ranged from 5.88 to 7.10 (Table 1). PO species tended to acidified the rhizosphere of soils A and B as it is shown in the results obtained in treatments without amendment addition. Soils A and B presented lower TOC content than soil C. For that reason, the effect of the amendment addition was only observed for soils with low organic matter content without a clear influence of the species.

In general, all studied soils presented low available Cd, especially in soils B and C (below the detection limit). Available Cu was increased by amendments, especially by

BC addition. Among species, ME and PO tended to mobilize more Cu than MA. The opposite was found for Zn, amendments reduced Zn availability although MA was the species that also mobilized less Zn (data no shown).

*3.2.2. Biomass, metal accumulation and distribution in plant tissues.*

Although amendments increased plant biomass, significant differences were not found between treatments in any case, except in MA in soil C (significant higher biomass in AC compared to CO). In this regard, ME was the species more positively affected by both amendments and, to a lesser extent, MA (Table 1). The increase of biomass found in ME and MA pots in the C amended soil was remarkable.

The effect of the amendments on metal uptake was different depending on the soil and plant species (Fig. 3). In soils A and B significant differences were only observed for PO and ME (not enough plant material was obtained for MA replicates). In soil A, significant differences for PO and ME were not observed, although all metal concentrations in PO shoots tended to increase due to the amendment addition, especially when BC was added. In soil B, significant lower concentrations due to amendment were only found for Cd in PO species. Finally, in soil C significant differences were found for Cu in ME and MA species due to amendments, and in Zn only for ME species (Fig. 3).

Among species, concentrations of Cd and Zn were significantly higher PO than in ME in soils A and B (statistic differences with MA could not be carried out due to the lack of replicates in soil A and B) (Fig. 3). In soil C, significant differences were found for the three species and elements (Cd, Cu and Zn, MA> PO>ME). According to the metal availability in the soils, Cd and Zn uptaken by all species were the highest in soil A, followed by the values obtained in soil B (here, particularly in case of Cd). Copper concentration was similar in the three studied soils despite the availability in C soil was lower than in A and B soils.

*3.2.3. Low molecular weight organic acids.*

The effect of soil type on exudates amounts was clear being similar between soil A and B and completely different to soil C (Fig. 5). Oxalic acid was the only LMWOA that was released in the three soils and in the rhizosphere of the three species (Table S2). Fumaric acid was only measured in MA exudates in soil A and B (and in ME in soil C) at low concentrations (Fig. 5 and Table S2). Citric acid was only released by the roots of ME (in soil A and B) and of PO (in soil amended B soil). In the case of soil C (amended or not), concentration of LMWOAs for all the species was much lower than values obtained for the other two soils. In this soil, exudates composition did not differ between treatment-species combination except for ME growing in amended soils (Fig. 5), where four LMWOAs analysed were released.

In general, contents of oxalic and malic acids released for plant growing in soils soil A and B were higher than those obtained for plants growing in washed sand (Experiment 1). However, content of these acids measured in soil C was lower than those found in the experiment using washed sand (Fig. 2 and Table S2).

*3.2.4. Factorial Analysis and correlations.*

By means of Factorial analysis, we established the importance of “Soil” (Fact 1) and “Plant species” (Fact 2) in this study (Fig. 6). In non-amended treatments (CO), factor 1 (35.2% of variance) was described by TOC and LMWOAs (except fumaric acid) whereas factor 2 (34.3%) was explained by metal concentrations in plant and fumaric acid. There was a clear difference between soils, mainly with soil C (highest TOC content and lowest LMWOAs exudates). The plant species factor played also an important role in this separation. MA species showed a strong relation with metal in plant and the release of fumaric acid in contrast to PO and ME. However, ME was the species most affected by Soil factor. Characteristics of soil C (higher organic matter than the other soils) made that the differences between species were smaller than in the other soils.

The application of organic amendments entailed changes in the arrangement of factor scores and, therefore in the importance of factors in each case, especially with the *alperujo* compost (AC). In this case, factor 1 (41.7% variance) became more relevant than in control. Factor 1 was explained, in addition to TOC, by oxalic and malic acid and metal uptake (Cd and Zn), whereas the variables described by factor 2 (24.8%) were citric and fumaric acid. Factor scores were mainly arranged along a single axis (factor 1). In this case, the metal uptake and the concentrations of malic and citric acids released into the rhizosphere were directly related between them and opposed to the TOC content, which established the difference between the 3 studied soils. In treatments applying biosolid compost (BC), factor 1 (32.7%) was defined by TOC, oxalic and malic acids whereas metal uptake and fumaric acid were related to factor 2 (32.4%). Citric acid was partially related to both factors (Fact 1: 0.668; Fact 2: -0.574).

The correlation analysis showed that, for all species, TOC was inversely correlated with Cu and Zn available, and with LMWOAs concentration ($p<0.05$). Correlation between metals bioavailability and organic acids also depended on the plant species. The availability of Cu in the soils was positively correlated with an increase of malic acid (PO and MA $p<0.01$; ME $p<0.05$) and with oxalic and fumaric acids (in MA) ($p<0.01$). However, fumaric acid content decreased with the increase of Cu and Zn availability in ME rhizosphere ($p<0.01$). In the case of PO and MA, LMWOAs were positively correlated with Zn availability (PO $p<0.01$; MA $p<0.05$). In general, metal content in plants were positively correlated with oxalic and malic acid. However, correlations between metal concentrations in aerial parts and fumaric acid were found positive for MA and negative for ME.

## 4. Discussion

For the three studied species (PO, ME and MA) the LMWOAs detected were oxalic, malic, citric and fumaric acids in all plant exudates (except specific cases). In particular, the main LMWOAs released (in both experiments) were oxalic and malic acid in agreement with previous studies carried out in contaminated environments with other plant species (Zeng et al., 2008; Quartacci et al., 2009). The scarce exudation (and in many occasions the absence) of fumaric acid measured in root exudates can be related to stress type (resulting from metal presence) since malic, citric and oxalic have a higher involvement in the complexation of metals (Hinsinger et al., 2006). Several factors can affect plant exudates e.g. plant nutrient status (Bowsher et al., 2015), metal stress, soil type (pH, organic matter content, soil structure) and plant species (Chiang et al., 2006). These acids are also released in response to low nutrient availability (Dakora and Phyllips, 2002) but as the nutritional requirements were covered in washed sand medium, the root exudates only responded to the stress derived of contamination without other factors interfering. In this medium, citric acid was the LMWOA that clearly reflected the effect of the contamination gradient tested, due to its increase at high doses, independently of the species. The biomass decreased and the highest metal concentrations in plants at high doses coincided with the citric acid increase which could be related to the ability of citric acid to promote the movement of heavy metals in the rhizosphere (mainly Zn and Cd) at near neutral pH (Schwab et al., 2008; Ding et al., 2014).

In absence of soil, the organic acids composition and quantity depend mainly on plant species and metal doses (Meier et al., 2012). The experiment carried out in washed sand allowed to study metal plant uptake of each species, because this substrate reduces the environmental variables that can affect/modify metal mobility. Metal mobility, and hence the metal concentration that can be uptaken by plants, may vary in the rhizosphere according to the species (Montiel-Rozas et al., 2015). The different plants behavior could be related to the strategy that each plant species develops in heavy metal contaminated environments (Bao et al., 2011). In this study, the three studied plants tended to exclude metals until moderate contamination rates (until dosis 4). However, at higher doses MA accumulated higher metal concentrations in their tissues than the other plants. Although at higher concentrations of Cd, Cu and Zn a significant increase of oxalic acid was observed, the highest metal accumulation in MA concurred with the presence of lowest levels of LMWOAs. This is in agreement with previous findings that established the higher exudation of LMWOAs as an efficient exclusion mechanism reducing the metal uptake and allowing the plant growth at high levels of contamination (Meier et al., 2012). The concentrations of organic acids in ME exudates (a leguminous species) were the highest (Fig. 2) and, in MA, the lowest, composed mainly by oxalic acid. This result is related to the LMWOAs production increment as a plant mechanism against toxicity generate by metals due to the chelating character of organic acids (Han et al., 2006; Xu et al., 2007). Malic acid concentration in PO (a graminaceae species) exudates was, together with oxalic acid, relevant at all contamination levels. Cadmium and Cu concentrations found in PO roots (the lowest concentrations found in all species) could be related to the possible role of oxalic and malic acids alleviating phytotoxicity under metal stress (Zeng et al., 2008). Another factor that could explain the lower metal concentration in *P. annua* roots is the secretion of phytosiderophores (amino acids) which form stables complex with metals (Hinsinger et al., 2006).

Different factors affect to plant exudates when plants grow in soils. In this study, the low differences between pH values in the soils allowed to evaluate the relation of organic acids with other variables as Total Organic Carbon and study plant behavior without a decisive influence of pH which has a strongly effect on heavy metal extraction/availability and on organic acids (types and concentrations) (Ding et al., 2014). In the studied soils, a clear effect of amendments on soil pH was not detected. However, the effect of amendments in the amount and composition of LMWOAs was different depending on the plant species, increasing in treated soils growing PO and decreasing in the case of ME exudates. Modification of production and composition of plant exudates because of amendment application has been established in previous studies (Koo et al., 2006; Park et al., 2011).

Soil factor was more important since there were clear differences between LMWOAs composition found in contaminated soils (Fig. 6). In soil with higher organic matter content (soil C), with the exception of ME in amended soils, only oxalic acid was found in the exudates. In the other soils (A and B), more similar in terms of organic matter content, the composition of the exudates was similar.

Soil C showed a better nutritional level than Soil A and B. As it has been said above, LMWOAs were also released in response to low nutrient availability (Dakora and Phyllips, 2002), which could explain the lower contents of oxalic acid and no malic excretion in this soil. Particularly, malic acid excretion has been reported as a plant response in phosphorus deficient conditions (Chang et al., 2002, Bais et al., 2006).

The organic carbon content in soils was directly related to the decrease of metal availability in the rhizosphere due to its ability to complex metals (Park et al., 2011).

Subsequently, the reduction of metal stress results in a decrease in quantity and diversity in organic acids exudates. Numerous studies have shown the important role that the LMWOAs play on bioavailability and mobility of the metals in soils (e.g. Hinsinger et al., 2006; Haoliang et al., 2007). Acidification or modification of redox conditions and chelating ability are the mechanisms to change the metal and nutrient availability (Seshadri et al., 2015). In the present study, the higher Cu availability in soils with lower organic matter content was related to the exudation of malic acid. The efficacy in Cu desorption from soil particles showed by malic acid is due to high stability of malate-metal complexes (Qin et al., 2004). Moreover, it should be noted that the level of Cu in aerial parts of plants varies scarcely between soils, despite the availability of this element was much lower in soil C.

Addition of amendments also leads to a biomass increase, although PO was the species less affected. Nevertheless, high biomass values in all cases were found in soil with higher organic matter (soil C). Thereby, plant growth conditions were better due to a reduction of stress conditions and results in a similar organic acid exudation of all species. The amendments also supplied nutrients and among the amendments applied, *alperujo* compost reduced differences between species in soils A and B. This effect could be related to the intrinsic characteristics of amendment since they provide higher concentrations of nutrients and less heavy metals than biosolid compost (Madejón et al., 2014).

## 5. Conclusions.

The assessment of LMWOAs exudates in two different matrixes has allowed to know the potential response of each plant species to exposition at different Cd, Cu and Zn concentrations (both in metal uptake and LMWOAs exudation) as well as the behavior in real conditions under different heavy metal stress levels.

Both composition and amount of LMWOAs vary according to the species and the soil conditions. In addition to metal availability, nutrient level as well as organic carbon content have a direct effect on the plant and therefore on its exudates. Due to different tolerance to metal stress and physiology, plant exudates differed although the main organic acids released by all species were oxalic and malic acids. Both organic acid concentration and composition were related to metal concentration in vegetal tissues. Thereby, the leguminous (*M. polymorpha*) was the species with lowest metal concentrations in tissues, highest values of biomass and a more diverse composition of organic acids exudates. Effect of amendments on plant exudates varied according to the species. The concentration of LMWOAs exudates by *P. annua* roots increased in amended treatments whereas *M. polymorpha* exudates decrease (mainly with *alperujo* compost addition). To conclude, the exudation of LMWOAs has demonstrated to be an important response mechanism of plants to phytotoxicity caused by heavy metals since increasing their excretion at high contamination levels. Moreover, there is also an important influence of the soil quality, in terms of organic matter and nutrient contents, because the highest LMWOAs concentrations were produced under poor soil quality conditions.

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**Figure captions.**

**Fig. 1** A) Dry biomass (g) (significant differences are indicated through the text) and B) heavy metals concentrations metals in shoots and roots for each specie grown in washed sand at each contaminant dose. PO, *P.annua*; ME, *M. polymorpha*; MA, *M. sylvestris.* Significant differences between species for each doses and element are marked as follows: ***, differences were found between 3 species; **PO/**ME/**MA differences between specie indicated and the others species; differences between two species are indicated with the initial species and * (e.g. PO*ME)

**Fig. 2** Mean values (Standard errors, n=3) of LMWOAs (mmol $h^{-1}$) for each doses and plant species. Significant differences are indicated through the text

**Fig. 3** Factor scores corresponding to different plant species and contaminant doses applied are arranged along two main factors . Variables used in the Factorial analysis (Biomass; Low Molecular Weight Organic Acids; Cd, Cu and Zn in roots and shoots) are marked in black colour. *P. annua*, Circles; *M. polymorpha*, Square; *M. sylvestris*, Triangle

**Fig. 4** Cadmium, Cu and Zn in aerial part of plants grown in the three studied soils (A, B and C). Significant differences for each soil and plant per treatment are indicated with different letters. CO, No Amendment; BC, Biosolid compost; AC, Alperujo compost; PO, *P. annua*; ME, *M. polymorpha*; MA, *M. sylvestris*

**Fig. 5** Heatmap clustering. Heatmap shows the different exudates (LMWOAs) composition according to the different treatments, plants and soils. For each organic acid, values are colored from clear yellow (low) to red (high).

**Fig.6** Factor scores corresponding to different plant species and soils are arranged along two main factors. Factorial analysis was carried out for the different treatments (Control, *Alperujo* and Biosolid compost) and the variables used (Total Organic Carbon; Low Weight Molecular Organic Acids; Cd, Cu and Zn uptake by plant) are represented in black colour. *P.annua*, Circles; *M.polymorpha*, Square; *M.sylvestris*, Triangle; Soil A, red colour; Soil B, blue colour; Soil C, green colour

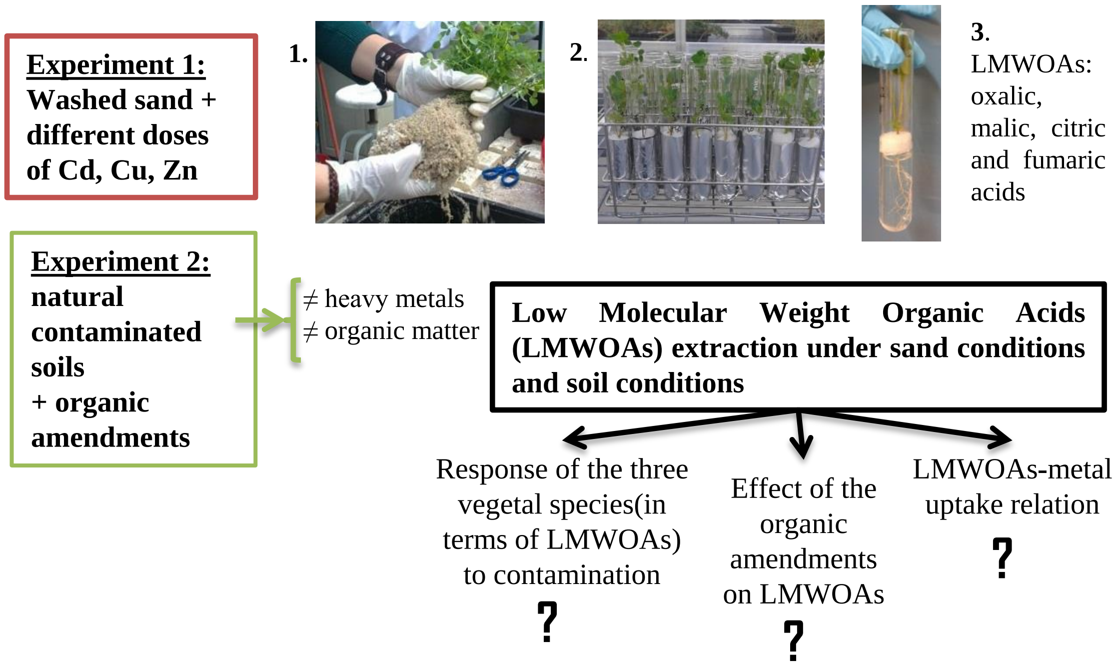
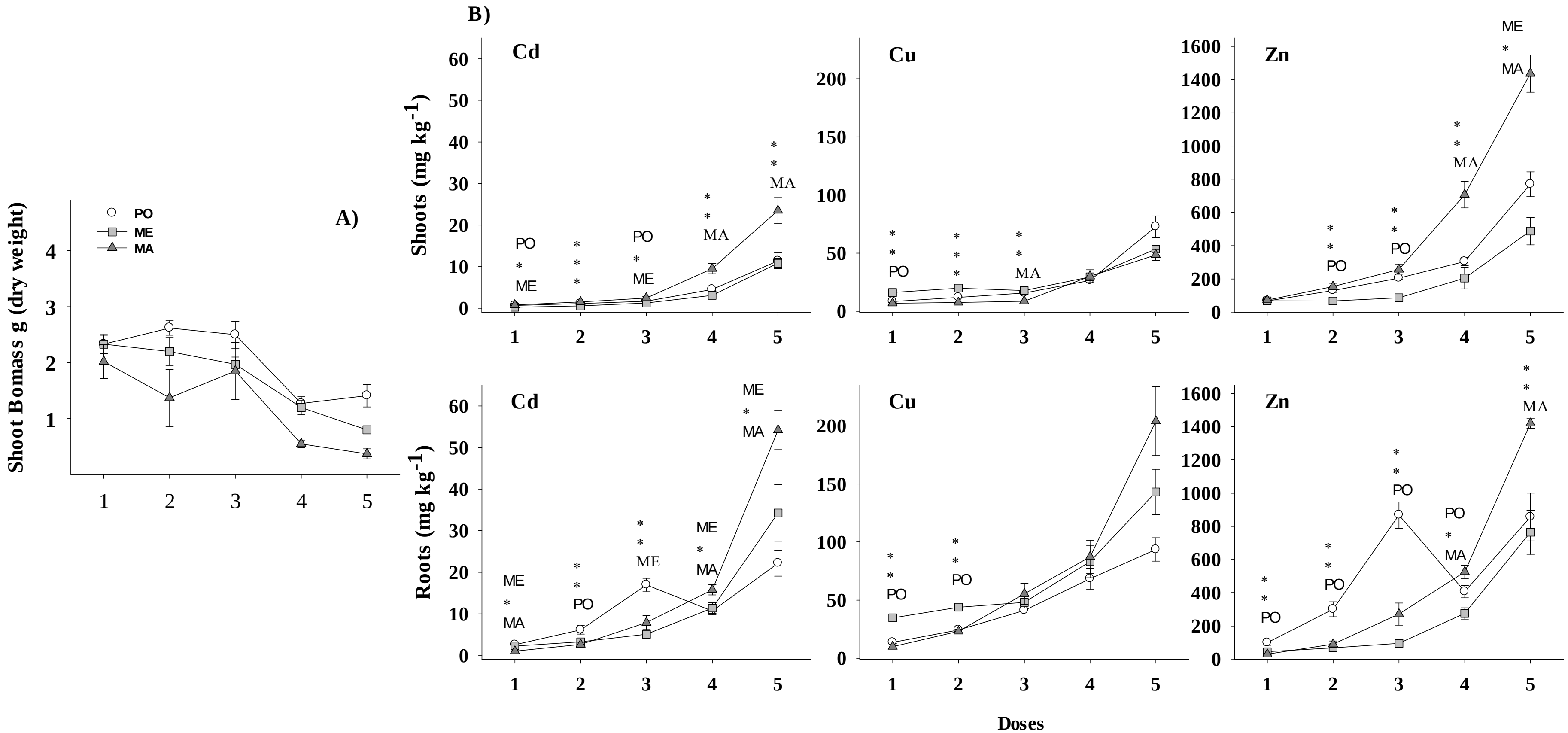

Experiment 1:
Washed sand + different doses of Cd, Cu, Zn
1.
2.
3.
LMWOAs: oxalic, malic, citric and fumaric acids
Experiment 2:
natural contaminated soils
+ organic amendments
≠ heavy metals
≠ organic matter
Low Molecular Weight Organic Acids (LMWOAs) extraction under sand conditions and soil conditions
Response of the three vegetal species(in terms of LMWOAs) to contamination
?
Effect of the organic amendments on LMWOAs
?
LMWOAs-metal uptake relation
?

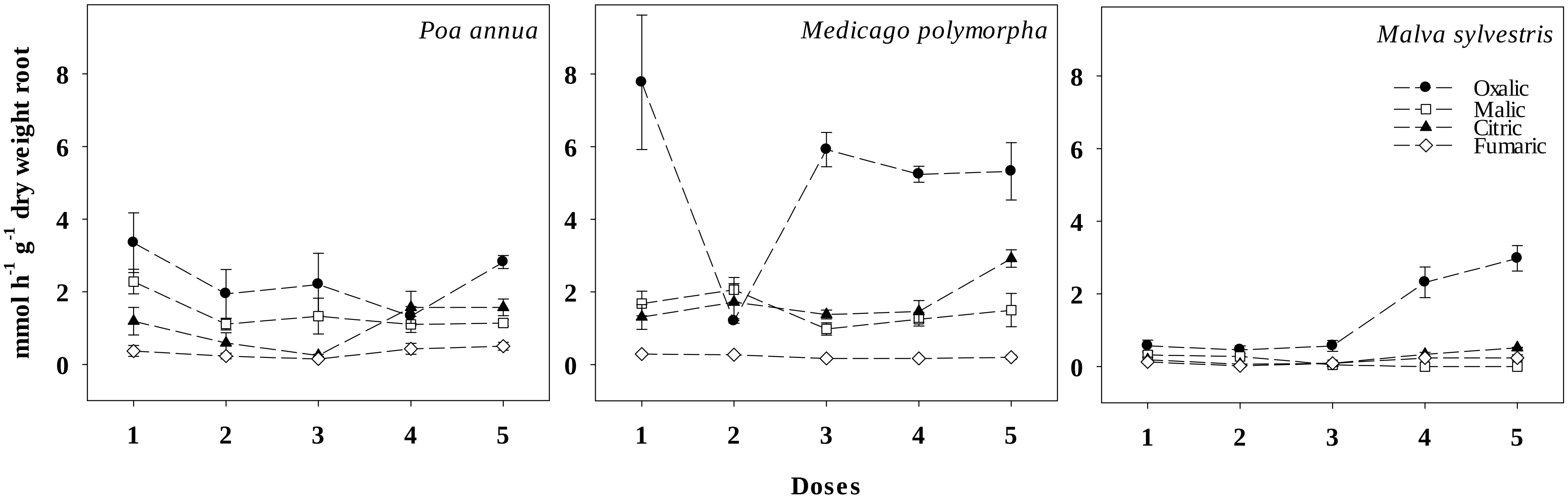
Table 1. Mean values of pH, Total Organic Carbon (TOC) and biomass by species for each treatment and soil. Significant differences between species per soil and treatment are indicated by different letters (p<0.05). CO, No Amendment; AC, Alperujo compost; BC, Biosolid compost; PO, *P.annua*; ME, *M. polymorpha*; MA, *M. sylvestris.* Standard errors in parenthesis

| | | **pH** | | | **TOC (g kg⁻¹)** | | | **Shoot biomass (g)** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *PO* | *ME* | *MA* | *PO* | *ME* | *MA* | *PO* | *ME* | *MA* |
| **A** | **CO** | 7.10 (0.03)a | 6.99 (0.00)b | 6.99 (0.01)b | 18.5 (1.0) | 18.2 (0.7) | 16.2 (0.4) | 0.76 (0.23)ab | 1.89 (0.52) b | 0.08 (0.01)a |
| | **AC** | 6.92 (0.01) | 6.91 (0.03) | 6.97 (0.02) | 21.3 (1.7) | 22.8 (1.6) | 18.9 (0.8) | 1.33 (0.26)ab | 3.07 (0.78)b | 0.4 (0.13)a |
| | **BC** | 6.88 (0.02) | 6.85 (0.01) | 6.96 (0.05) | 28.6 (1.4)a | 24.2 (1.4)ab | 20.5 (1.7)b | 0.64 (0.18)a | 2.85 (0.47)b | 0.27 (0.09)a |
| B | **CO** | 6.50 (0.08)a | 5.93 (0.05)b | 5.88 (0.03)b | 6.3 (0.7)a | 9.6 (0.4)b | 7.8 (0.3)c | 0.53 (0.04)ab | 1.45 (0.61)b | 0.03 (0.01)a |
| | **AC** | 6.20 (0.07)a | 6.46 (0.04)b | 6.51 (0.02)b | 11.9 (1.7)ab | 15.2 (0.5)a | 11.0 (0.3)b | 0.57 (0.14) a | 3.10 (0.43) b | 0.13 (0.03)a |
| | **BC** | 6.44 (0.06) | 6.57 (0.04) | 6.59 (0.04) | 10.9 (0.4) | 14.0 (0.4) | 14.9 (2.2) | 0.51 (0.08)a | 2.80 (056)b | 0.06 (0.03)a |
| C | **CO** | 6.14 | 5.9 | 6.1 | 53 (3.7) | 52.1 (1.6) | 52.6 (1.4) | 1.90 (0.15)a | 1.71 (0.22)a | 1.54 (0.28)a |
| | **AC** | 6.61 | 6.47 | 6.38 | 55.8 (3.8) | 60.5 (2) | 60.3 (1.3) | 1.67 (0.32)a | 5.23 (1.10)ab | 6.01 (1.52)b |
| | **BC** | 6.76 | 6.52 | 6.57 | 65.2 (2.7) | 60.7 (1.5) | 55.3 (0.4) | 2.15 (0.14)a | 5.04 (1.61)a | 3.94 (0.52)a |



A)
PO
ME
MA
Shoot Bomass g (dry weight)
B)
Cd
Cu
Zn
Shoots (mg kg-1)
Roots (mg kg-1)
Doses


Figure 1



Poa annua
Medicago polymorpha
Malva sylvestris
Oxalic
Malic
Citric
Fumaric
mmol h-1 g-1 dry weight root
Doses


Figure 2

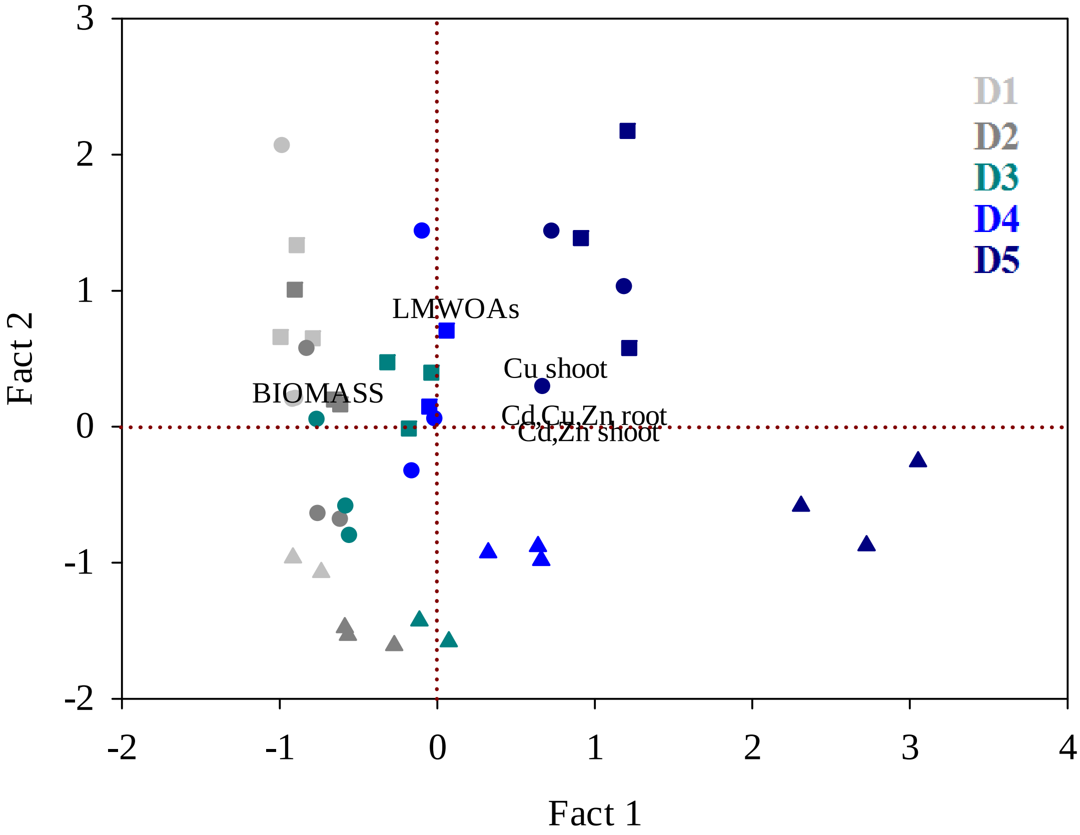
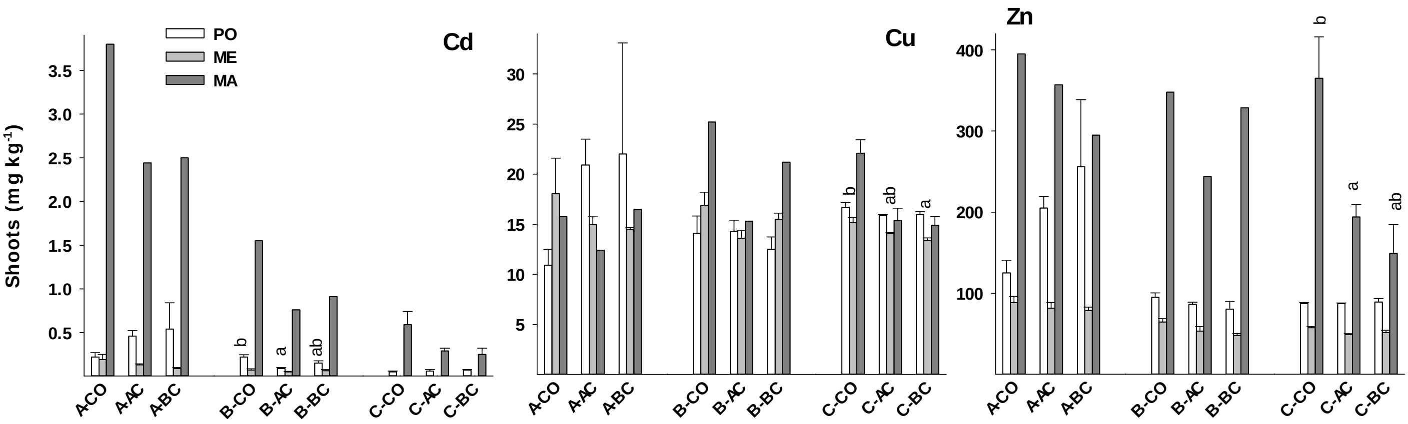

D1
D2
D3
D4
D5
LMWOAs
Cu shoot
BIOMASS
Cd,Cu,Zn root
Cd,Zn shoot
Fact 2
Fact 1
3
2
1
0
-1
-2
-2
-1
0
1
2
3
4

Fig. 3



Cd
Cu
Zn
PO
ME
MA
Shoots (mg kg-1)
A-CO
A-AC
A-BC
B-CO
B-AC
B-BC
C-CO
C-AC
C-BC


Figure 4

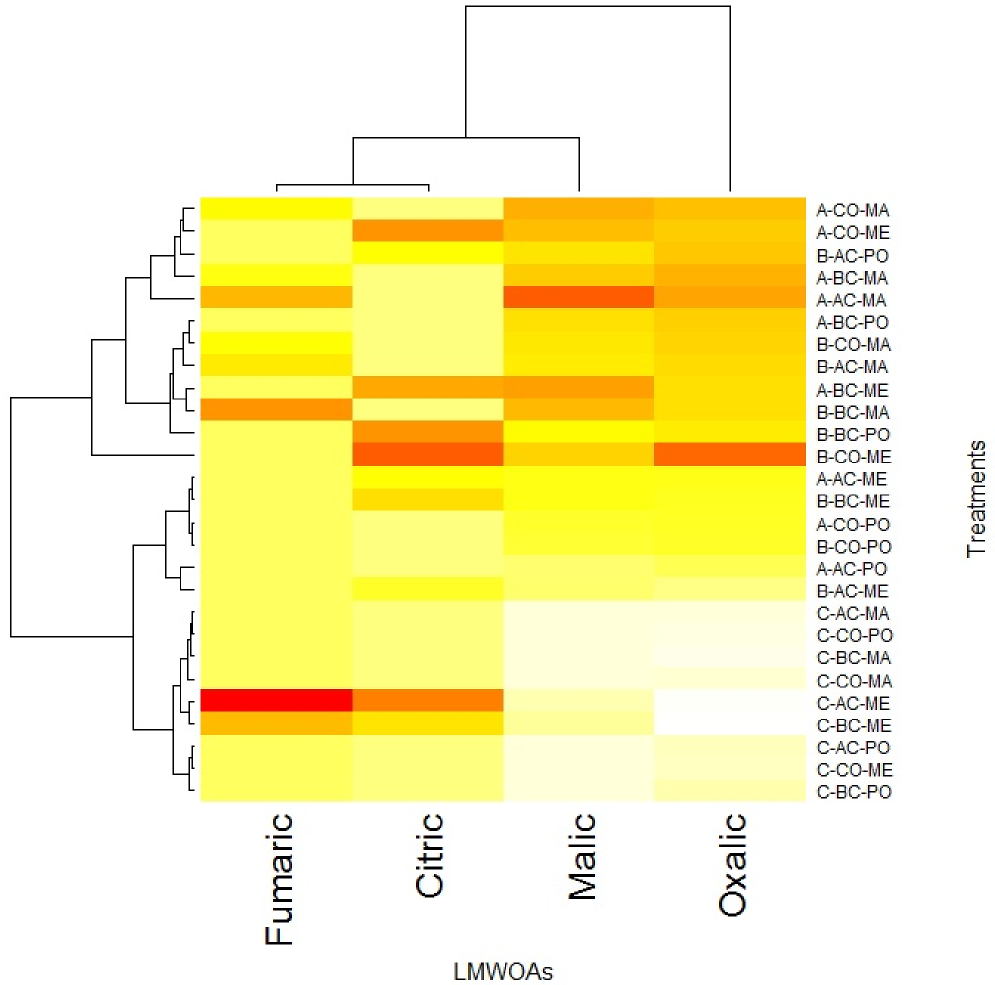

A-CO-MA
A-CO-ME
B-AC-PO
A-BC-MA
A-AC-MA
A-BC-PO
B-CO-MA
B-AC-MA
A-BC-ME
B-BC-MA
B-BC-PO
B-CO-ME
A-AC-ME
B-BC-ME
A-CO-PO
B-CO-PO
A-AC-PO
B-AC-ME
C-AC-MA
C-CO-PO
C-BC-MA
C-CO-MA
C-AC-ME
C-BC-ME
C-AC-PO
C-CO-ME
C-BC-PO
Treatments
Fumaric
Citric
Malic
Oxalic
LMWOAs

Figure 5

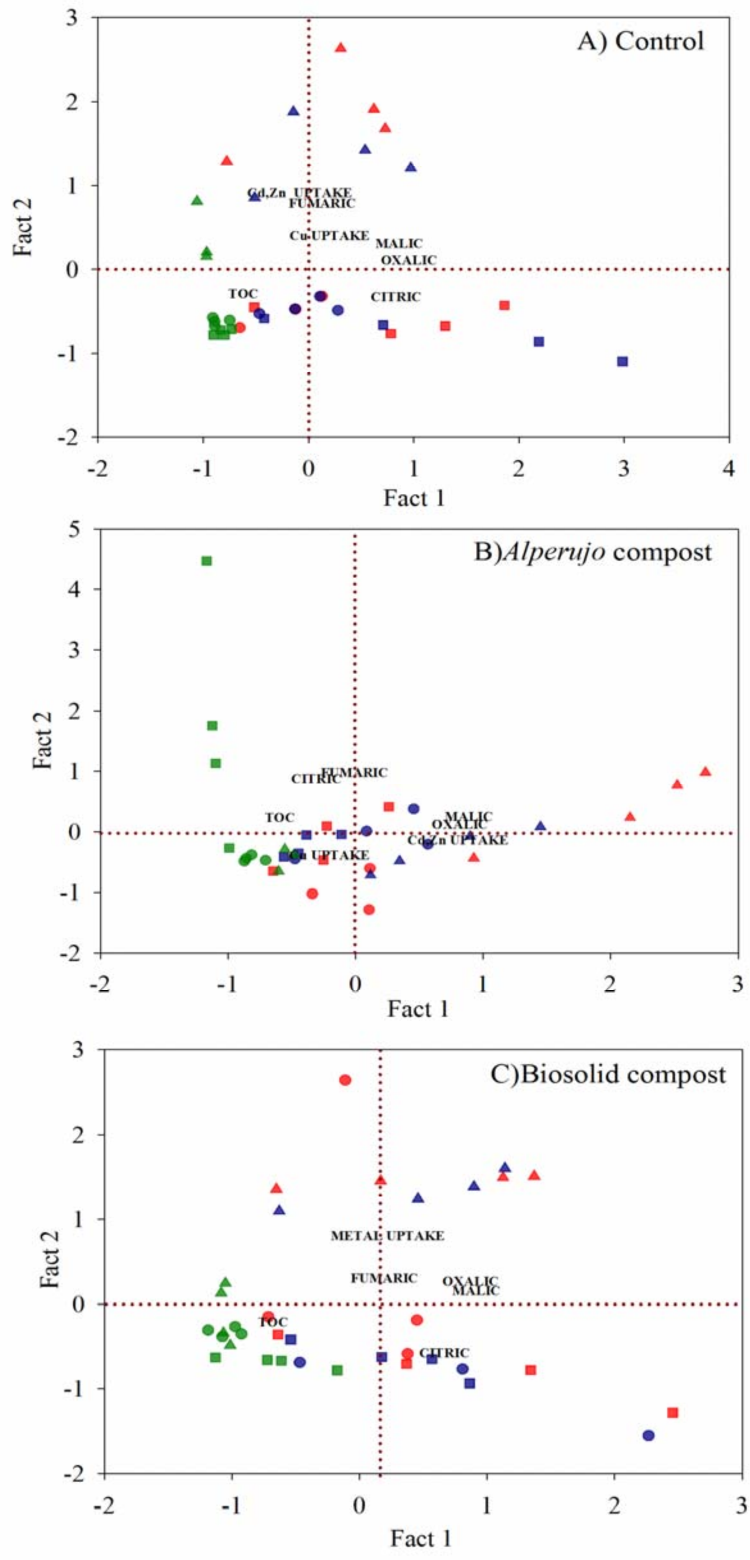

A) Control
Cd,Zn UPTAKE
FUMARIC
Cu-UPTAKE
MALIC
OXALIC
TOC
CITRIC
Fact 1
Fact 2
B)*Alperujo* compost
CITRIC
FUMARIC
TOC
MALIC
OXALIC
Cd,Zn UPTAKE
Cu UPTAKE
Fact 1
Fact 2
C)Biosolid compost
METAL UPTAKE
FUMARIC
OXALIC
MALIC
TOC
CITRIC
Fact 1
Fact 2

Figure 6