

THE EFFECTS OF MYCORRHIZAL FUNGI, STREPTOMYCETES AND PLANTS ON HEAVY METAL MOBILITY AND BIOACCUMULATION IN AN INDUSTRIALLY ENRICHED SOIL: PRELIMINARY RESULTS OF A LYSIMETER EXPERIMENT

Nicoara A., Neagoe Aurora, Donciu Roxana, Iordache V.

University of Bucharest, Faculty of Biology, Research Center for Ecological Services - CESEC

ABSTRACT

We performed a lysimeter experiment to observe the influence of mycorrhizal fungi and streptomyces amendments on heavy metal mobility and plant uptake in a soil enriched with Pb (around 2000 mg/kg), Cu (around 250 mg/kg) and Zn (around 500 mg/kg) by atmospheric deposition from a battery factory near Bucharest, Romania. This study shows the results of the first stage in a series of four alternative Helianthus annuus and Secale cereale cultures on contaminated soil amended with Glomus intraradices fungi and Streptomyces acidiscabies and S. tendae bacterial inocula. Soil metal concentrations, Eh, moisture, temperature, pH and nutrient variation have been correlated with plant oxidative stress and microbial activity in an attempt to explain the influence of bacteria and fungi inocula on soil conditions and plant bioaccumulation.

Key words: *lysimeter, heavy metals, Helianthus annuus L., bioremediation*

INTRODUCTION

Heavy industrialization in the communist period has led to the significant enrichment in heavy metals of soils in and near urban areas in Romania, with the highest concentrations near metal extraction and processing centers (Lucaciu et al. 2004, Pope et al. 2005, Damian et al. 2008). Although most of the industrial processing installations generating atmospheric plumes or leachate have been closed or moved in recent times their close proximity to living quarters, highly frequented institutions and water sources, and the persistent nature of heavy metal pollutants in the soil mean that they still pose a threat to the environment and human health (Pope et al. 2005, Lăcătușu 2010). The former industrial areas east of Bucharest, the capital and largest city in Romania are hot spots of heavy metal enrichment of soils through atmospheric deposition and material/waste storage. Their close proximity to settlements causes heavy metals to enter local water systems through leaching into the water table and rivers, leading to noticeable health hazards, such as elevated lead concentrations in the blood of children in the area (Velea 2009).

In this context of persistent enrichment of certain areas with heavy metals, phytoremediation methods, that use the potential of plants and microorganisms to reduce the negative influence of these areas on humans and other organisms gain more and more interest (Kabata-Pendias 2001, Pilon-Smiths 2005, 2006, Vangronsveld 2009) as they are less disruptive and sometimes cheaper due to less extensive logistics (Neagoe et al. 2006). Heavy metals may exit from an enriched soil via either leaching or bioaccumulation, paths determined by solubility and

bioavailability, which are in turn controlled by plant cover, microbial activity, organic matter and clay content, Eh, pH, drainage and mineral composition of the soil (Kabata-Pendias 2001, Lordache et.al. 2006). Rhizosphere microorganisms influence metal mobility in the soil by changing soil conditions, like pH, Eh and organic matter content, secretion of chelating agents, accumulation and adsorption or by specific or non-specific biotransformation (Tabak 2005, Wenzel 2008). Moreover the rhizosphere is host to complex relationships between plants, their associated mycorrhiza and bacteria, forming a network of interactions and substance exchanges known as the wood-wide web, linking many different individuals and insuring fast nutrient transfer and protection from toxic elements (Giovanetti 2006, Bonfante 2009). To know and stimulate these interactions, where they have been disrupted by man's influence, gives the possibility to direct remediation techniques for optimal results in regard to available financial resources and decisional factors' interest in area reclamation. Also, knowledge gathered from mezoscale experiments is useful in perfecting models of metal mobility at full scale in software applications (Lordache et.al. 2009), giving a powerful prediction tool to aid in the full scale directing of phytoremediation towards extraction or stabilisation of metals.

MATERIALS AND METHODS

Soil.

Soil for the lysimeter installation was sampled from about 4km east of Bucharest, in the Pantelimon area that has been an industrial platform for decades. The main source of heavy metals enrichment was atmospheric deposition from the "Neferal" factory's chimney plume. The factory specialized in battery production and non-ferrous metals, since 1932. After 1995 the factory's activities were limited to recycling used batteries into lead, aluminum, and other non-ferrous material products. The soil near the chimney of the factory is richest in heavy metals, in concentrations close to 2000 mg/Kg for lead, 250 mg/Kg for copper and 500 mg/Kg zinc, with the highest concentrations at a depth of 0-15 cm. Soils predominating the area are reddish-brown preluvosoils, characterized as moderately acid, with a clayey texture, having small to average humus contents (1.8 – 3.7%) and total nitrogen (0,130 – 0,163%) and small amounts of soluble phosphorus (11 – 15 mg/kg) (Lăcătușu 2008).

Experimental setting.

The lysimeter installation (Figure 1.) consisted of 10 undisturbed soil monoliths, 30 cm wide by 60 cm tall, sampled from the same area to reduce heterogeneity.

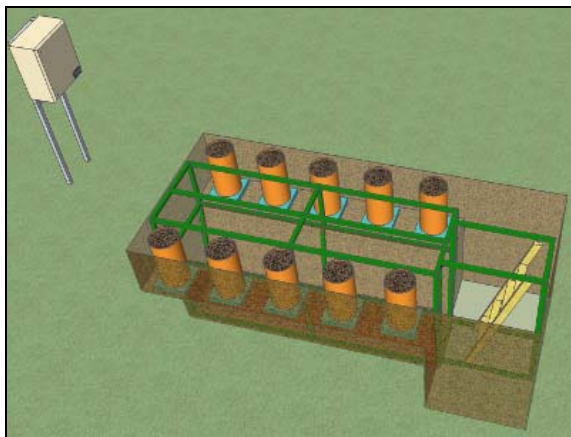


Figure 1 Lysimeter installation.

The top layer of herbaceous plants and dense roots (ca. 5 cm of soil) was removed prior to sampling. The lysimeters were equipped with temperature, redox and humidity sensors and data was constantly monitored with a datalogger. Field tension was simulated at the bottom of the monoliths with a vacuum pump, also used to sample leachate. The installation was housed in a below-ground chamber for thermal insulation purposes. Plant cultures consist of 4 alternate successions of rye

(planted in autumn, harvested in summer) and sunflower (planted in mid-summer and harvested in autumn). Results will only be shown for the first culture of sunflower. Experimental variants consisted of 2 unamended, negative control replicates, 4 replicates amended with *Glomus intraradices* mycorrhizal inoculum in expanded clay (10%, mixed in the first 20 cm of soil), and 4 replicates amended both with *G. intraradices* and the streptomycetes: *Streptomyces acidiscabies* and *Streptomyces tendae* in liquid CSA growth medium, 10 ml per lysimeter.

Soil and water analyses.

We analyzed key parameters for soil, water, plants and microorganisms. Soil analysis was performed at sampling in the immediate vicinity of each of the lysimeters from 0 to 60 cm depth in 10 cm increments, and after each plant harvest at 0 – 15 and 15 – 30 cm depths. Soil moisture was calculated after drying soil samples at 105°C until constant weight. pH was measured in a soil water mixture (1:2.5). Soil samples were kept at 4°C and processed within 24 hours after sampling. For nitrogen compounds, 20 g of soil were extracted with 100ml 0.2M KCl solution and for phosphate 5 g with 100ml 0.5 M NaHCO₃. Samples were analyzed through colorimetric methods: ammonium by Na nitroprussid, nitrate by sulphosalicylic method, nitrite N1 naphthylethyldiamine and sulphanilimide and phosphate with molid-ammonium and malachite green (Neagoe et al. 2005). Elements were analyzed on an Elan DRC-e ICP-MS from Perkin Elmer after digestion with aqua regia using an Anton Paar Multiwave 3000 digestion oven.

Leaching water was sampled after major rain events, leachate volumes and metal concentrations in leaching water were recorded.

Plant analyses.

After harvesting, plants were measured weighed and separated into roots, shoots leaves and flowers. Roots were washed in tap water, distilled water and deionized water. Plant material was freeze dried, ground and stored at -45°C. Fresh and freeze-dried biomass and individual heights were recorded.

For protein and enzyme assays, dry plant material (50 or 100mg) was homogenized in 4ml cold 100mM potassium phosphate buffer containing 2% polyvinylpyrrolidone, 2mM EDTA and 2mM dithioerithrol and centrifuged at 6000 rpm for 20 minutes at 4°C. The supernatant was dialyzed overnight at 4°C in 5mM K phosphate buffer. Protein concentrations were determined spectrophotometrically with alkaline copper reagent and Folin-Ciocaltau reagent against a BSA standard curve (Lowry 1951, Iordachescu 1980). Superoxide dismutase was measured through the inhibition of the rate of reduction of Cytochrome c by the superoxide radical, observed at 550 nm according to McCord and Fridovich (1969). Peroxidase activity was determined by spectrophotometrically measuring the transformation of guajacol to tetraguajacol in the presence of H₂O₂ according to Mascher et al (2002). the reaction mixture contained 33mM guajacol and 0,3mM H₂O₂ in 50 mM citrate/phosphate buffer.

For the chlorophyll and carotenoid assay, 50 mg of dry plant matter was homogenized in a buffer containing 80% acetone, 15% water and 5% NH₃ 25% solution. Samples were then centrifuged to remove solids and spectrophotometrically measured at 480, 638, 645, 647, 663 and 664 nm and chlorophyll a, chlorophyll b and carotenoids were measured as described by Schöpfer (1989).

Lipid peroxide tests were performed according to Heath and Packer (1968)

: 20 mg of dry biomass was homogenized with 4ml TBA buffer containing 10% trichloroacetic acid and 0.25% thiobarbituric acid in ultra-pure water, heated for 30

min at 95° C, cooled for 15 min at room temperature, centrifuged and spectrophotometrically measured at 532 and 600nm.

Microorganism analysis.

Root fragments were cleared with KOH and colored with lactophenol blue for mycorrhiza differentiation. Roots were divided into approximately 1cm long fragments and around 20 fragments from each experimental variant were observed under a Nikon microscope.

Statistic analyses

Statistic test were performed with the software “Statistica”.

RESULTS AND DISCUSSIONS

Plant biomass.

Biomass differed consistently between replicates (Figure 2). One of the unamended negative reference lysimeters (R1) and two of the replicates amended only with fungi (F2 and F4) showed very poor growth compared to other replicates of the same experimental variants. Differences in biomass between the negative reference variant and the one amended with *G. intraradices* mycorrhizal fungi were not statistically significant because of this high variation. Biomass differences between negative reference and fungi plus *S. acidiscabies* and *S. tendae* variant were close to statistic significance ($p = 0.064$), but still non-significant due to the usage of only 2 replicates for negative reference (due to financial constraints).

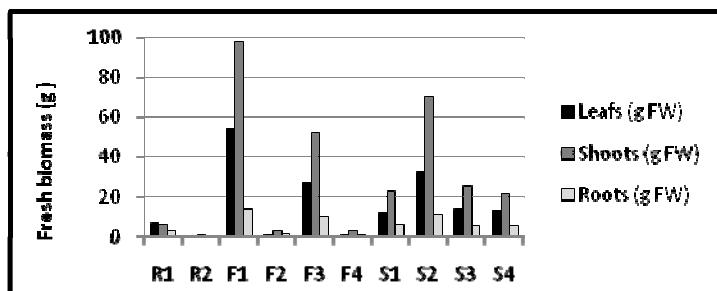


Figure 2 Plant biomass. R = unamended reference F = amended with mycorrhizal fungi S = amended with fungi and streptomycetes

Next, we shall focus on finding an explanation for the poor performance of the three replicates from the data provided by the studied parameters.

Plant health correlated with metal concentrations in plant tissue.

We focused mainly on Pb, Zn and Cu, as they were the major contaminants in the studied area. We were unable to corelate differences between mean plant tissue metal concentrations and stress for unamended reference against fungi amended and fungi and streptomycetes amended variants due to insufficient biomass for one of the R variants, however comparing the latter two yielded significant results. Most important, there were lower metal concentrations in the roots of fungi and streptomycetes amended replicates, but other parameters also varied in a significant manner (Figure 3).

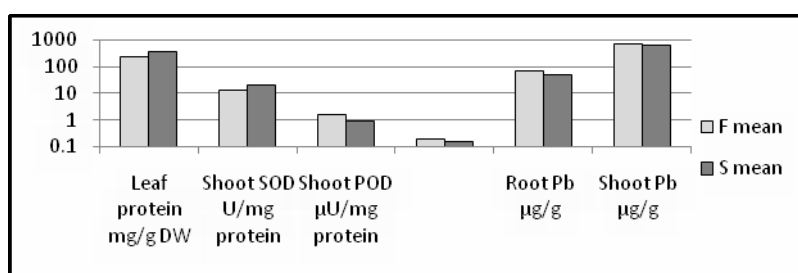


Figure 3 The variation of metal concentrations and oxidative stress enzymes between F and S variants. F = amended with mycorrhizal fungi S = amended with fungi and streptomycetes

We were also able to find a negative correlation between metal and

assimilating pigment concentrations when comparing fungi amended and fungi plus streptomycetes amended variants (Figure 4).

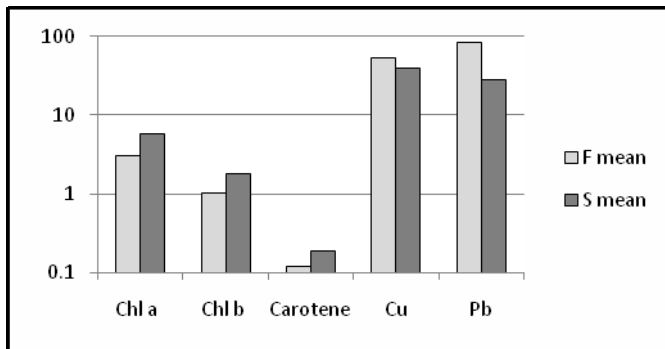


Figure 4 Correlation between metal concentrations and assimilating pigments between F and S variants.

F = amended with mycorrhizal fungi
S = amended with fungi and streptomycetes

When investigating metal accumulation for each of the replicates we observed a linear relationship between logarithmic values of biomass and metal concentrations, suggesting metal accumulation did not increase with biomass, rather a dilution of metals in plant tissue occurring for plants with more biomass (Figure 5). The distribution of accumulated heavy metal concentrations closely mirrored that of biomass, again underlining the bad performance of the same three lysimeters.

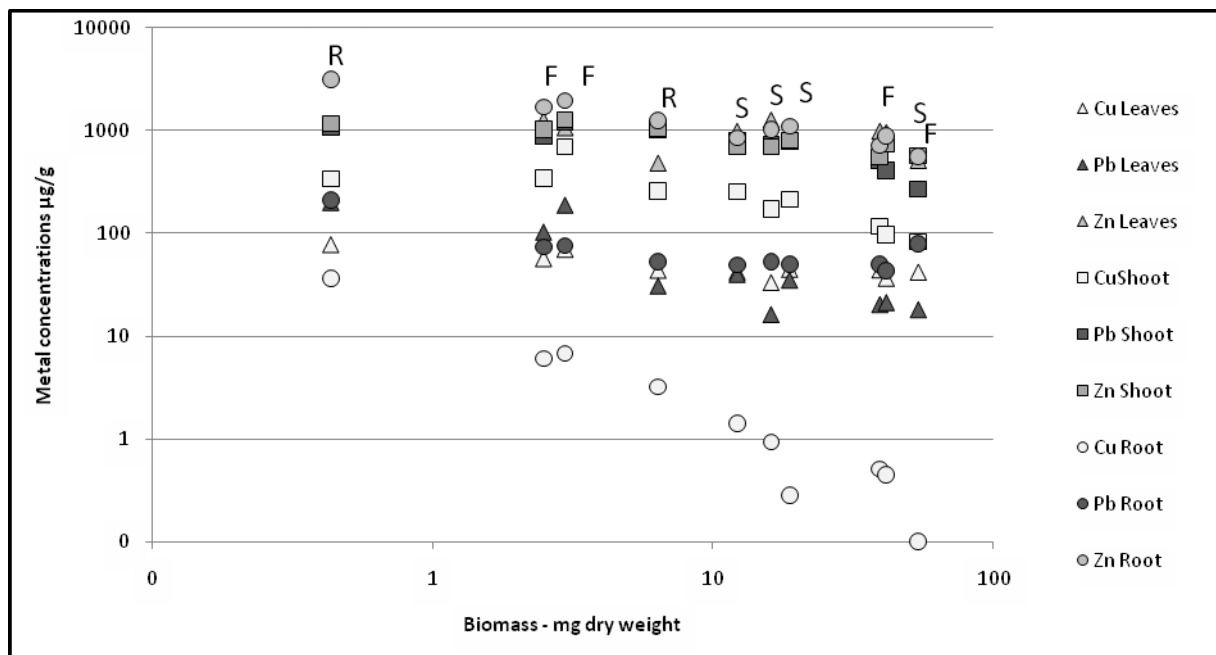


Figure 5 Linear logarithmic relationship between total biomass of each of the lysimeters and their plant metal concentrations. R = unamended reference, F = amended with mycorrhizal fungi, S = amended with fungi and streptomycetes.

Factors influencing metal mobility and plant uptake.

Next, we looked at other factors influencing heavy metal bioaccumulation, such as total soil concentrations, microbial activity, soil pH, metal solubility and leaching and redox potential of the soil.

There were insignificant differences in soil pH between replicates after harvesting. Microbial activity was slightly higher in replicates amended with streptomycetes, as expected (Figure 6).

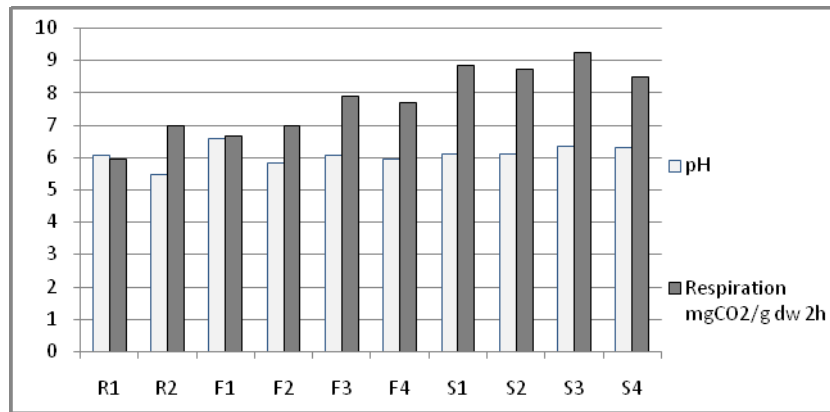


Figure 6 pH and microbial activity variation between experimental replicates after harvesting. R = unamended reference, F = amended with mycorrhizal fungi, S = amended with fungi and streptomycetes.

Pb, Cu and Zn soil concentrations in the soil at monolith sampling were decreasing with depth, but their variation pattern was not consistent with the three lysimeters with poor plant growth (Figure 7). After harvesting, concentrations of the same metals in the lysimeter soil also did not reveal a pattern explaining the dilemma (Figure 8). As total soil metal concentration were not the explanation, we turned to metal bioavailability. Parameters in the first 5 cm of soil prior to monolith sampling showed a high degree of heterogeneity, varying in all lysimeters and one parameter alone did not explain the poor growth of lysimeters R2, F2 and F4, but there might be complex conditions for plant inhibition due to different metal concentrations and small-scale microbial communities variation as suggested by initial nutrient data.

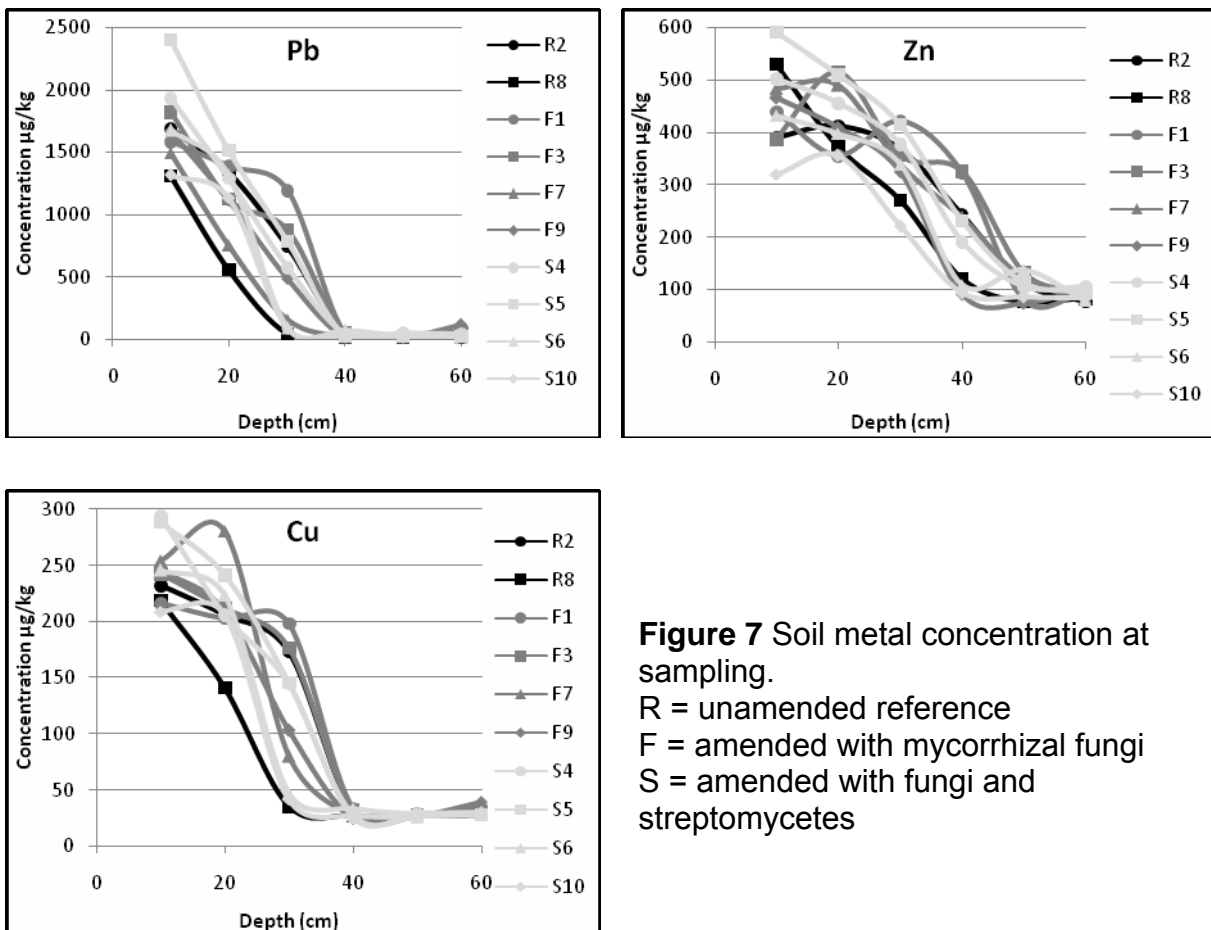


Figure 7 Soil metal concentration at sampling.
 R = unamended reference
 F = amended with mycorrhizal fungi
 S = amended with fungi and streptomycetes

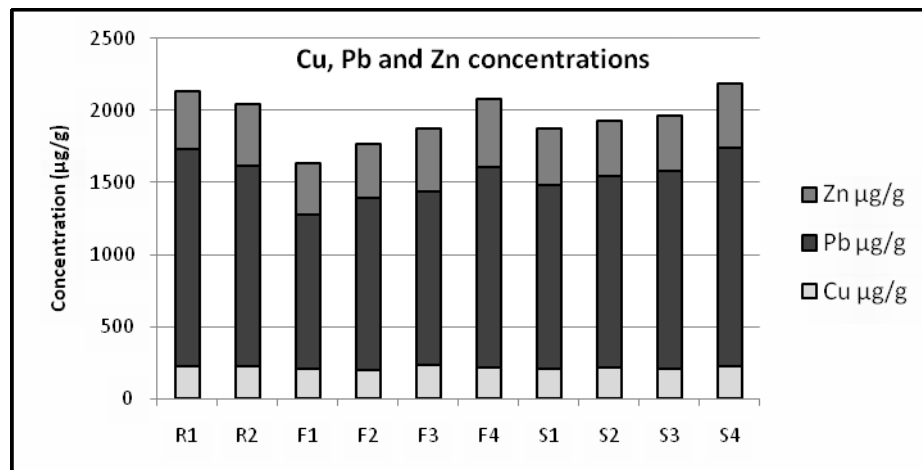


Figure 8 Soil metal concentrations at plant harvesting. R = unamended reference, F = amended with mycorrhizal fungi, S= amended with fungi & streptomyces

Metal solubility: metal exports through leaching

Lysimeter F2 had highest metal exports due to metal solubility and not higher permeability of substrate, showing highest metal mobility. R2 and F4, also poor growers, showed more metal solubility than F1 and F3, which had good plant growth (Table 1).

Table 1 Metal exports through leaching. R = unamended reference, F = amended with mycorrhizal fungi, S = amended with fungi & streptomyces

Total metals exported through rain				Tot. water	Mean metal conc. in leachate			
	Cu (µg)	Pb (µg)	Zn (µg)	(ml)		Cu (µg/l)	Pb (µg/l)	Zn (µg/l)
R1	25.35	44.90	5469	11550	R1	2.19	3.89	473.5
R2	27.60	66.38	5141	10650	R2	2.59	6.23	482.7
F1	21.79	34.36	3335	6715	F1	3.24	5.12	496.7
F2	32.00	75.66	5032	8630	F2	3.71	8.77	583.1
F3	16.33	22.41	2043	11800	F3	1.38	1.90	173.1
F4	26.17	31.51	4179	12300	F4	2.13	2.56	339.8
S1	22.04	35.16	4518	13150	S1	1.68	2.67	343.6
S2	15.97	25.43	4186	11250	S2	1.42	2.26	372.1
S3	26.79	52.34	4264	10700	S3	2.50	4.89	398.5
S4	19.27	23.20	4885	10825	S4	1.78	2.14	451.3

Monitoring data: humidity and redox.

A two month monitoring of humidity variation showed drainage differences between some of the lysimeters. Drainage patterns were homogenous amongst replicates, with a decrease of moisture dynamics with depth, explained by the slowing down of the water flow as it infiltrated deeper into the monoliths after wetting or a meteorological event. Some differences existed: lysimeter F2, one of those with plant growth problems showed higher humidity dynamic at 30 cm than at 10 cm, showing a possible preferential flow in that area. Also, lysimeter S2 showed

constantly higher humidity at 50 cm, an indication of possible drainage problems (Figure 9).

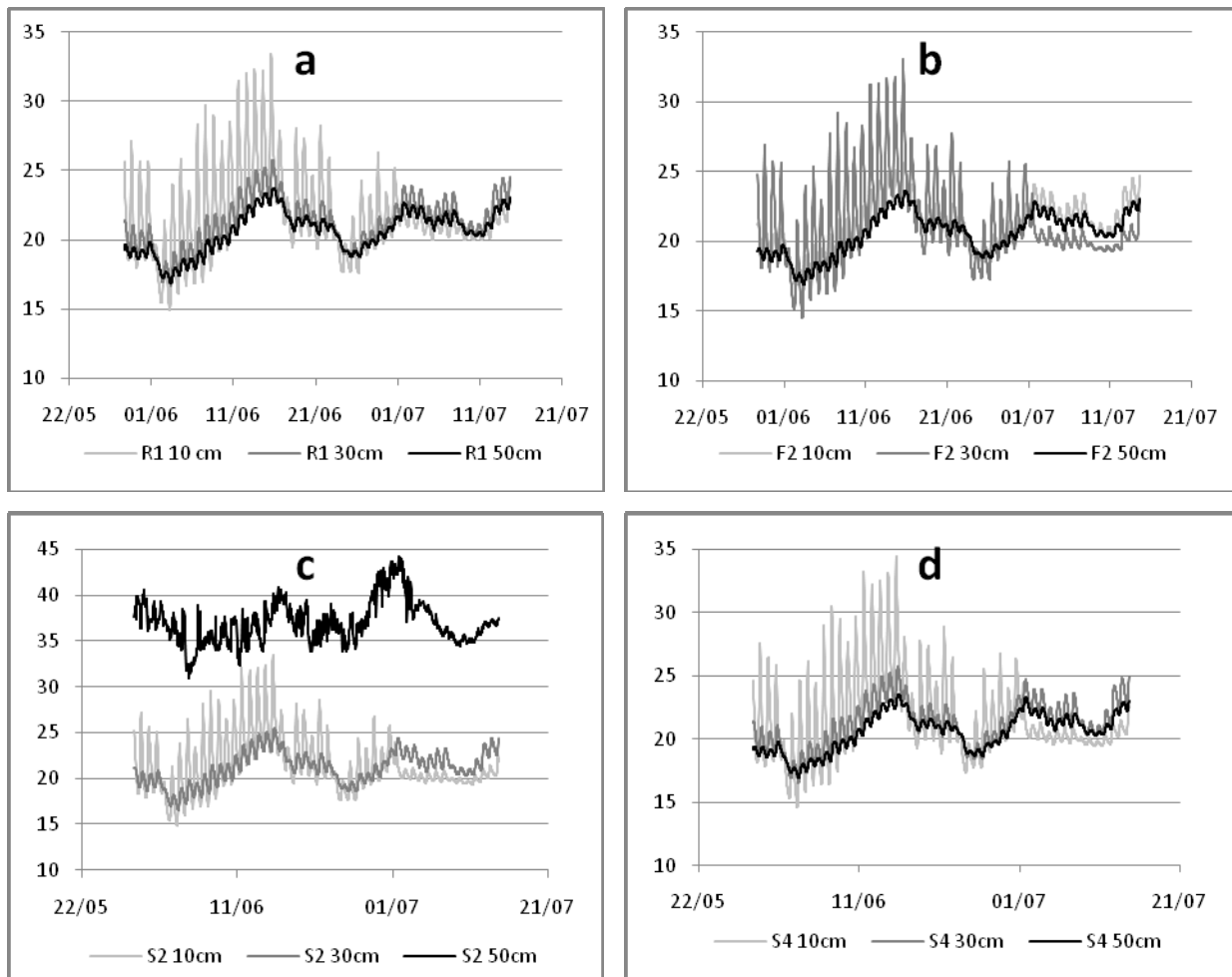


Figure 9 Humidity monitoring data. a. normal dynamic in negative reference lysimeter R1. b. possible preferential flow in fungi amended lysimeter F2. c. drainage problems in fungi and streptomycetes amended lysimeter S2. d. normal dynamic in lysimeter S4.

The monitoring of redox evolution yielded the most interesting results concerning the low biomass production of replicates R2, F2 and F4. These lysimeters manifested a far stronger drop in redox potential in the first 10 cm of soil at rain events than the other lysimeters (Figure 10).

Mycorrhization.

We were unable to calculate statistically significant mycorrhization degrees of plant roots due to the small biomass divided between the many lab tests. On the other hand, microscopic observations of colored root fragments revealed vesicles and arbuscules only in variants amended with mycorrhizal fungi inoculums, respectively F and S variants (Figure 11).

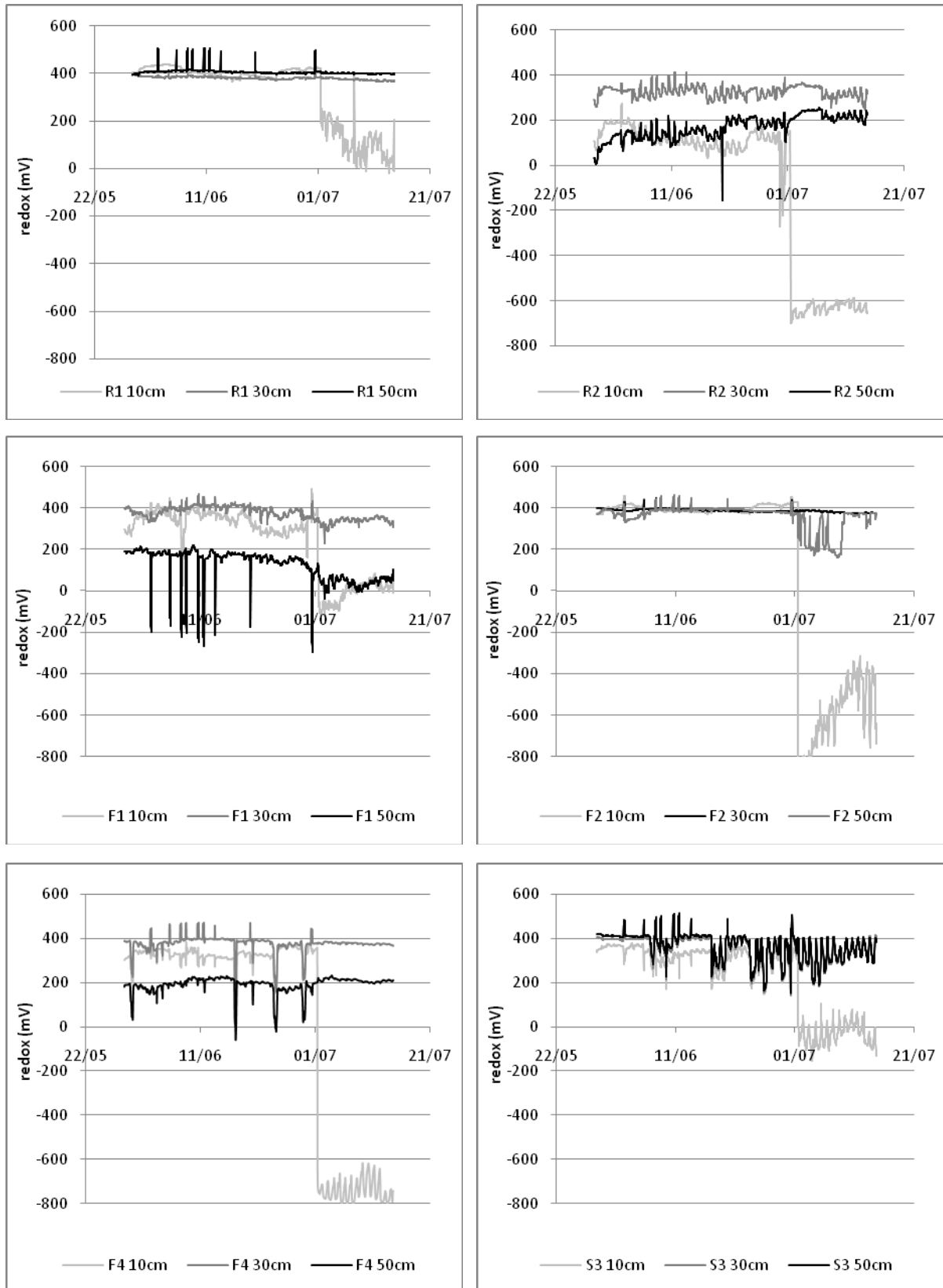


Figure 10 Redox monitoring data. Lisimeters R1, F1 and S3 showing a normal redox evolution. Lisimeters R2, F2 and F4 showing stronger drop in redox at rain events.

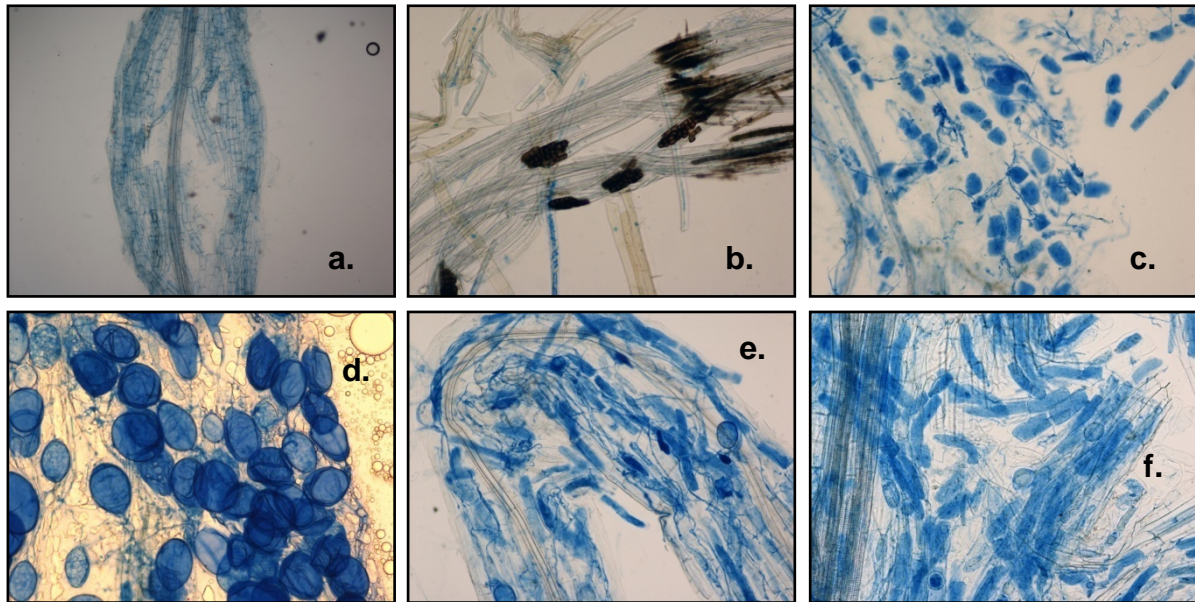


Figure 11 Microscopic images of colored root fragments. a. and b. fragments from R plants, showing no mycorrhiza, c. and d., fragments from F plants showing arbuscules and vesicles, e. and f., fragments from S plants showing arbuscules.

CONCLUSIONS

Using streptomycetes as an additional inoculum seems to significantly influence soil conditions, plant health and metal uptake.

As this is a multi-stage experiment, the data interpretation is limited to showcasing phenomena patterns within the different lysimeters, complete data interpretation being possible only after the cultures will be finished and all the data will have been gathered.

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