Environ Sci Pollut Res (2014) 21:7403–7412
DOI 10.1007/s11356-014-2696-z

RESEARCH ARTICLE

Inoculation with arbuscular mycorrhizal fungi and addition of composted olive-mill waste enhance plant establishment and soil properties in the regeneration of a heavy metal-polluted environment

Gustavo Curaqueo · Mauricio Schoebitz · Fernando Borie · Fuensanta Caravaca · Antonio Roldán

Received: 11 December 2013 / Accepted: 20 February 2014 / Published online: 4 March 2014

Abstract A greenhouse experiment was carried out in order to investigate the effects of arbuscular mycorrhizal (AM) fungi inoculation and the use of composted olive waste (COW) in the establishment of *Tetraclinis articulata* and soil properties in a heavy metal-polluted soil. The treatments assayed were as follows: AM+0 % COW, AM+1 % COW, and AM+3 % COW. The higher doses of COW in combination with AM fungi increased shoot and root biomass production of *T. articulata* by 96 and 60 %, respectively. These treatments trended to improve the soil properties evaluated, highlighting the C compounds and N as well as the microbiological activities. In relation to the metal translocation in *T. articulata*, doses of COW applied decreased the Cr, Ni, and Pb contents in shoot, as well as Cr and As in root, although the most of them reached low levels and far from phytotoxic. The COW amendment aided *Glomus mosseae*-inoculated *T. articulata* plants to thrive in contaminated soil, mainly through an improvement in both nutrients uptake, mainly P and soil microbial function. In addition, the combined use of AM fungi plus COW could be a feasible strategy to be incorporated in phytoremediation programs because it promotes soil properties, a better performance of plants for supporting the stress in heavy metal-contaminated soils derived from the mining process, and also can be a good way for olive-mill waste disposal.

Responsible editor: Stuart Simpson

G. Curaqueo · F. Borie
Scientific and Technological Nucleus of Bioresources, BIOREN-UFRO, Universidad de La Frontera, P.O. Box 54-D, Temuco, Chile

M. Schoebitz (✉) · F. Caravaca · A. Roldán
CSIC-Centro de Edafología y Biología Aplicada del Segura, P.O. Box 164, Campus de Espinardo, 30100 Murcia, Spain
e-mail: mschoebitz@gmail.com

Keywords Polluted soils · Mining · Organic amendment · Enzyme activities · Phytostabilization · Soil remediation

Introduction

Mining activities affect relatively small areas but can have a large spatio-temporal impact on the environment due to the deposition of residues at the surface close to the mining area (Wong 2003). In this sense, mine tailings are the primary component of mine waste after ore-processing for metal extraction, and are considered as one of the main risks in post-mining landscapes due to the generation of a high amount of waste material (Conesa et al. 2007; Mendez and Maier 2008) resulting in a high degradation of the environmental quality (Adriano et al. 2004) and human health (Morais et al. 2012). The tailings are composed mainly of silt or sand-sized particles and have poor soil stability (Mendez and Maier 2008), high salinity, low fertility (Norland and Veith 1995), scarce organic matter (Kabas et al. 2011), strong acidification/alkalinization processes (Conesa et al. 2006), and an important amount of heavy metals which are accumulated in the soil (Kabas et al. 2011; Conesa et al. 2006).

The rehabilitation of contaminated land by mining activities is often a complex and costly process (Ciccu et al. 2003), but in the last decades, there have emerged a large number of strategies to remediate the environmental damage caused by mining activities (Ciccu et al. 2003), including the use of physical, chemical, or biological mechanisms (Meier et al. 2012). Among the alternatives available, the use of vegetation for remediation of heavy metal-polluted soils can be an attractive option (Tordoff et al. 2000).

An important aspect to be considered in the revegetation processes of heavy metal-contaminated soils is understanding

plant–microorganism interaction, such as arbuscular mycorrhizal (AM) fungi. AM fungi are plant symbionts widely distributed in terrestrial ecosystems (Smith and Read 2008) including soils polluted with high metal levels (Azcón et al. 2009; da Silva et al. 2006; del Val et al. 1999). AM fungi establish associations with the majority of plant species (Jeffries et al. 2003) and are fundamental in optimizing plant fitness and soil quality. Mycorrhizal symbiosis improves the resilience of plant communities against environment stresses, including nutrient deficiency, drought, and soil disturbance (Barea et al. 2011). In heavy metal-polluted soils, AM fungi play a fundamental role to alleviate heavy metal stress of plants, helping for the revegetation in these mining areas (Hildebrandt et al. 2007). Another alternative widely used to stimulate the revegetation process in the contaminated soils is the application of stabilizing soil amendments which can improve plant growth (Azcón et al. 2009; Fernández et al. 2012), and also can enhance key biological processes affecting the immobilization of heavy metals (Adriano et al. 2004; Houben et al. 2012) resulting in an improved quality of soil properties (Córdova et al. 2011; Pagliai et al. 2004). In the same way, diverse studies have demonstrated the beneficial effect of the application of organic amendments and AM fungi in diverse ecosystems (Gosling et al. 2006; Oehl et al. 2004; Valarini et al. 2009) including polluted soils (Alguacil et al. 2011).

The Spanish olive-mill industry produces a huge amount of wastes difficult to reuse (4 million ton per year). The addition of olive-mill wastes to contaminated soils could influence physicochemical soil properties and fertility status to improve phytostabilization in polluted soils. After composting, these wastes are non-phytotoxic, rich in K, and partially humified organic matter, and have low levels of heavy metals (Alburquerque et al. 2006). Such characteristics suggest that this compost could be useful for improving soil quality parameters in the development of remediation programs for metal-contaminated soils, especially when considered together with the inoculation with beneficial microorganisms for revegetation purposes. The effectiveness of composted olive-mill waste in the restoration of the chemical and biological properties of heavy meta- contaminated semiarid soils (Alburquerque et al. 2011) and in the establishment of tolerant grasses such as *Bituminaria bituminosa* (Martínez-Fernández and Walker 2012; Pardo et al. 2013) or agricultural species (Fornes et al. 2009) has been recently reported. However, to date, there are no studies on the combined use of this organic amendment and the inoculation with AM fungi in the implantation of Mediterranean woody species in phytoremediation tasks.

The aim of this study was to provide alternative practices to solve the serious environmental problem of olive-mill wastes disposal and polluted soils regeneration. We hypothesize that the combined effect of AM fungi and composted olive-mill waste can effectively enhance both plant establishment and soil properties recovery during the phytostabilization of heavy metal-polluted soils derived from mining activities.

Materials and methods

Site description and test soil

Soil for this study comes from La Unión Mining District at Southeast Spain (Lat. 37° 35′ 39″ N, Long. 0° 52′ 57″ W). The area presents a low lying (<400 m above sea level) with steep slopes between 20–30 %. This area, which covers a surface of about 50 km^2, was an important mining nucleus in Spain in the last centuries. The ore deposits of La Unión contain iron, lead, and zinc as the main metal component. Fe forms oxides, hydroxides, sulfides, sulfates, carbonates, and silicates, whereas that Pb and Zn are present in galena, sphalerite, carbonates, sulfates, and Pb- or Zn-bearing (Mn, Fe) oxides (Oen et al. 1975). The climate is Mediterranean-semiarid with a mean annual temperature of 17.5 °C, an annual rainfall of around 250–300 mm, while the potential evapo-transpiration reaches 1,000 mm $year^{-1}$. The natural vegetation is formed mainly by small formation of *Pinus halepensis* and shrub plants with xerophytic characteristics. The soil sampling was conducted at one mine tailing called "El Gorguel" (U.TM. X687480 Y4162800 Z135; length, 200–300 m; width, 95 m; height, 25 m; volume, 750,000 m^3). Soil samples consisted of a homogeneous mixture of six sub-samples randomly collected from the top 20-cm depth of soil. Some analytical characteristics of the soil mine tailing are shown in Table 1.

Materials

A composted olive-mill waste (COW) was used as organic amendment for soil. Fresh cow bedding was added to the olive-mill waste as bulking agent and composted by using a combination of the Rutgers system and mechanical turning. This material showed mainly a basic pH, high organic matter content, and an elevated electrical conductivity (Table 1). The mycorrhizal inoculum was a mixture of rhizosphere soil containing spores, hyphae, and mycorrhizal root fragments. This material was originated by isolated spores from the soil in the experimental area and classified as *Glomus mosseae* (Nicol. and Gerd.) Gerdemann and Trappe, being the most abundant AM fungi ecotype in this area (Azcón et al. 2009). The mycorrhizal inoculum was multiplicated in trap cultures using *Sorghum bicolor* (L.) Moench as host plant. The plant used in this experiment was *Tetraclinis articulata* (Vahl) Mast. in J. Roy, a coniferous tree member of the *Cupressaceae* endemic of Northern Africa and Southeastern Spain (Sierra de Cartagena), where it is commonly used in revegetation programs. Previous to experimental procedures, plants were grown

Table 1 Physical, chemical, and microbiological properties of the soil and compost used in the experiment

Parameter	Soil	Composted olive-mill waste
pH (H_2O)	7.67±0.03	8.83
Electrical Conductivity (1:5, dS m^{-1})	1.34±0.76	6.12
Organic matter (%)	1.81±0.02	73.09
Total organic C (g kg^{-1})	10.5±0.1	438.6
Total N (g kg^{-1})	1.33±0.05	31.7
Total Cr (μg g^{-1})	91±3	12
Total Cu (μg g^{-1})	163±6	45
Total Fe (μg g^{-1})	190,000±5,100	1,810
Total Ni (μg g^{-1})	15±1	10
Total Mn (μg g^{-1})	5,900±200	104
Total Ca (μg g^{-1})	7,900±600	29,700
Total Mg (μg g^{-1})	11,900±400	5,700
Total Na (μg g^{-1})	273±6	4,100
Total K (μg g^{-1})	560±26	42,500
Total P (μg g^{-1})	319±19	568
Total Zn (μg g^{-1})	12,000±300	158
Available P (μg g^{-1})	7±1	–
Total Pb (μg g^{-1})	6,900±200	–
Total S (μg g^{-1})	12,700±300	–
Total Al (μg g^{-1})	14,500±300	–
Total Cd (μg g^{-1})	37±1	–
β-glucosidase (μmol PNP g^{-1} h^{-1})	0.01±0.01	–
Dehydrogenase (μg INTF g^{-1})	8.5±1.3	–

under nursery conditions for 10 months using peat as substrate. Before transplanting, plants reached (mean±SE, n=5) 44.3±3.2 cm height, 2.68±0.36 g shoot dry weight, and 0.78 ±0.17 g root dry weight.

Experimental design and setup

The experiment consisted of a completely randomized design with five replicates, where the factor evaluated was the addition of three different doses of composted olive-mill wastes to the contaminated soil from the mining area (without COW application; COW 1 % (*w*/*w*) and COW 3 % *w*/*w*). In addition, a mycorrhizal inoculum (*G. mosseae*) was used at a rate of 5 % (*v*/*v*) in all treatments. The experiment on *T. articulata* was conducted under greenhouse conditions. Individual plants were transplanted to 10-L plastic pots (20×45 cm, further called mesocosms) containing the different treatments (AM + 0 % COW, AM+1 % COW, AM+3 % COW). A control treatment without AM inoculation or COW amendment was also established but plants did not survive and only lasted a few weeks after transplanting. During the experiment, the plants were watered with sterile water until soil reached 60 % of field capacity. Twenty-four months after the transplanting, the plants were harvested; separating shoots and roots, and the soil was collected for analysis. Roots were abundantly washed with tap water to eliminate soil particles and quickly dried on paper before analysis.

Plant analyses

To evaluate the response to AM inoculation and COW application, the following growth parameters were evaluated: dry (105 °C, 48 h) weight of shoots and roots, basal stem diameter, and plant height was recorded before chemical analysis. Leaves and roots were ground after drying. About 1 g of both dry leaves and roots were burnt in a muffle during 24 h at 480 °C. Later, ashes were dissolved in HNO_3 (0.6 N) and filtered through an Albert® 145 ashless filter paper. This acid extract was used to measure shoots and roots metal contents and foliar P and K using an ICP-MS (Thermo Electron Corporation Mod IRIS Intrepid II XDL). The precision and accuracy of this method were tested by analyzing (five replicates) the CTA-VTL-2 certified material, corresponding to Virginia Tobacco leaves. The recoveries from plant standards ranged between 89–110 %. Foliar N was determined by dry combustion using a LECO Tru-Spec CN analyzer (LECO Corp., St. Joseph, MI, USA).

Fungal measurements

Arbuscular mycorrhizal root colonization was performed using a microscope (20–40×) after clearing a portion of the roots with 3 % H_2O_2 (*v*/*v*) and 10 % KOH (*w*/*v*) and staining in a 0.05 % solution of trypan blue in lactic acid (*w*/*v*). The gridline intercept method (Giovannetti and Mosse 1980) was used for determining the proportion of AM root colonization.

Mycelium density was measured according to Rubio et al. (2003). Briefly, 3 g of soil sample were mixed with a solution of glycerol/HCl/water (12:1:7 *v*/*v*), shaken for 30 min at 80 °C and the suspension filtered through 250- and 38-μm sieves. The material retained in the 38-μm sieve was re-suspended in 100-mL dH_2O, shaken for 1 min and allowed to stand for 30 s. Three milliliters of suspension were transferred to a membrane filter (0.45-μm pore size, 47-mm diameter, gridline interval 3 mm), stained with trypan blue solution (0.05 % *w*/*v*) and the hyphae were quantified under stereoscopic microscope at 100× (Giovannetti and Mosse 1980).

The easily extractable glomalin-related soil protein (EE-GRSP) was determined after taking 1 g of soil in 8 mL of citrate buffer (20 mM, pH 7.0) and autoclaving at 121 °C for 30 min (Wright and Upadhyaya 1998); then centrifuged at 10,000*g* for 15 min and filtered through Whatman No. 1 filter. The content of protein was determined by the Bradford protein assay (Bio-Rad Protein Assay; Bio-Rad Labs) with bovine serum albumin as standard (Wright et al. 1999).

Soil analyses

Soil pH and electrical conductivity were measured in a 1:5 (*w*/*v*) aqueous solution. The total organic C and total N were determined by dry combustion using a LECO Tru-Spec CN analyzer (LECO Corp., St. Joseph, MI, USA). The quality of the analytical procedure was checked using a reference material LECO Soil (#502-062). Total metal, phosphorus (P), and potassium (K) contents were determined by ICP-OES spectrometry (Thermo Elemental Co. Iris Intrepid II XDL). This methodology was referenced using the CRM027-050 Certified Material (Resource Technology Corporation, USA). The recoveries from soil standard were between 84–112 %. Water-soluble carbohydrates and total carbohydrates were determined by the method of Brink et al. (1960). Available P, extracted with 0.5-M $NaHCO_3$, was determined by colorimetry according to Murphy and Riley (1962).

Dehydrogenase activity was determined according to García et al. (1997). For this, 1 g of soil at 60 % of its field capacity was exposed to 0.2 mL of 0.4 % INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride) in distilled water for 20 h at 22 °C in darkness. The iodonitrotetrazolium formazan (INTF) formed was extracted with 10 mL of methanol by shaking vigorously for 1 min and filtering through a Whatman No. 5 filter paper. INTF was measured spectrophotometrically at 490 nm. Dehydrogenase activity was expressed in terms of microgram INTF per gram of soil with reference to a standard curve of INTF.

Urease and N-α-benzoyl-L-argininamide (BAA) hydrolyzing protease activities were determined in 0.1-M phosphate buffer at pH 7; 1-M urea and 0.03-M BAA were used as substrates, respectively. Aliquots of 2 mL of buffer and 0.5 mL of substrate were added to 0.5 g of sample followed by incubation for 90 min at 30 °C (urease) or 39 °C (protease). Both activities were determined as the $NH4^+$ released in the hydrolysis reaction (Nannipieri et al. 1980). The estimation of urease and BAA-protease activities were carried out using the standard curve of ammonium chloride.

Alkaline phosphatase activity was determined using p-nitrophenyl phosphate disodium (PNPP, 0.115 M) as substrate. For the assay, 2 mL of 0.5-M sodium acetate buffer adjusted to pH 11 using acetic acid (Naseby and Lynch 1997) and 0.5 mL of substrate were added to 0.5 g of soil and incubated at 37 °C for 90 min. The reaction was stopped by cooling at 2 °C for 15 min. Then, 0.5 mL of 0.5-M $CaCl_2$ and 2 mL of 0.5 M NaOH were added and the mixture centrifuged at 4,000 rev min^{-1} for 5 min. Absorbance at 398 nm was determined against the reagent blank and p-nitrophenol (PNP) content was calculated by referring to a calibration curve of PNP (Tabatabai and Bremmer 1969).

β-glucosidase was determined using 0.05-M p-nitrophenyl-β-D-glucopyranoside (PNG, 0.05 M) as substrate (Masciandaro et al. 1994). For this assay, based on the release and detection of PNP, 2 mL of 0.1-M maleate buffer at pH 6.5 and 0.5 mL of substrate were added to 0.5 g of sample and incubated at 37 °C for 90 min. The reaction was stopped with tris-hydroxymethyl aminomethano (THAM) according to Tabatabai (1982). The amount of PNP was determined by spectrophotometry at 398 nm (Tabatabai and Bremmer 1969).

For all enzyme assays, controls were included with each soil analyzed. The same procedure as for the enzymatic assay was followed for the controls but the substrate was added to the soil after incubation but prior to stopping the reaction.

The percentage of stable aggregates was determined by the method described by Lax et al. (1994). A 4-g aliquot of sieved (0.2–4 mm) soil was placed on a small 0.250-mm sieve and wetted by spray. After 15 min, the soil was subjected to artificial rainfall of 150 mL with energy of 270 J m^2. The soil remaining on the sieve was placed in a weighed capsule (T), dried at 105 °C for 8 h and weighed (P1). Then, the soil was soaked in distilled water for 2 h and passed through the same 0.250-mm sieve with the assistance of a small stick to break up the remaining aggregates. The residue remaining on the sieve, which was made up of plant debris and sand particles, was dried at 105 °C and weighed (P2). The percentage of stable aggregates was calculated as $(P1-P2)\times 100/(4-P2+T)$.

All physicochemical and chemical analyses were performed in duplicate and biochemical analyses in triplicate;

the results are expressed on a dry weight basis (24 h at 105 °C).

Statistical procedures

Prior to statistical analysis, data were log transformed to achieve for normality, the percentage of root colonization and stable aggregates were arcsin transformed. The data were statistically analyzed using one-way ANOVA and the means were compared using the Duncan test ($P \leq 0.05$). All statistical analyses were performed with the SPSS software v. 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Plant parameters

The use of higher COW (3 % *w*/*w*) amendment combined with *G. mosseae* inoculation significantly increased by 75 % the height of *T. articulata*. In the same way, the production of shoot and root dry matter increased by 96 and 60 %, respectively, with the use of this treatment while the biomass production was not significantly increased by the application of COW 1 % (Table 2). The addition of both COW doses significantly increased the foliar P but not the foliar K nor N concentrations in relation to non-amended plants.

Soil physicochemical analysis

The addition of COW significantly increased total soil C and N as well as the total soil organic carbon content and carbohydrates, whereas other soil parameters such as pH, electrical conductivity, and the percentage of soil particles aggregation showed no significant differences among treatment assayed (Tables 3 and 4).

Mycorrhizal parameters

The AM fungi colonization of *T. articulata* by *G. mosseae* increased by 50 % when the treatment of COW 3 % was applied; however, there were no significant differences. In turn, the AM mycelium density was not affected by the COW application, although a trend to increase the mycelium density with the use of this amendment was observed (Table 4). The glomalin contents presented significant differences among treatments, increasing the content of this glycoprotein with the use of higher doses of COW compared to the soil only inoculated with *G. mosseae*.

Soil enzyme activities

The application of COW-yielded significant effects on urease and protease-BAA activities, increasing 6.8 and 2.5 times their activity with the application of the highest dose of compost. In the same way, phosphatase activity showed a significant response with the use of the highest dose of COW, increasing by 34 %, while the use of the lowest COW dose increased this activity only by 13 % compared to the soil only inoculated with *G. mosseae*. β-glucosidase activity increased significantly with the application of both doses of composted olive-mill waste, while only the higher COW application dose produced significant increases in the dehydrogenase activity (Table 5).

Metal concentrations in soil and plants

The total metal contents in the rhizosphere soil were not significantly affected by the treatments evaluated (Table 6).

The application of the amendment significantly decreased the shoot concentrations of Cr, Ni, and Pb, without differences between doses. In root tissues, only As and Cr concentrations were significantly reduced by the organic amendment. It is of note that total metals and As concentration in roots highly

Table 2 Plant growth parameters, and foliar N, P, and K concentrations of *T. articulata* grown in a heavy metal-polluted soil treated with composted olive-mill waste and inoculation with *G. mosseae* ($n=5$)

Treatment	Basal stem diameter (mm)	Height (cm)	Shoot dry (g)	Root dry (g)	Foliar N (μg g^{-1})	Foliar P (μg g^{-1})	Foliar K (mg g^{-1})
AM	18.1±2.1	50.6±3.5b	48.5±4.7b	64.4±5.5b	0.55±0.03	0.64±0.11b	5.04±0.16
AM + COW 1 %	13.7±3.1	61.4±4.7b	43.4±4.3b	58.2±7.1b	0.53±0.02	1.94±0.20a	5.08±0.64
AM + COW 3 %	15.6±1.6	88.4±4.8a	95.2±4.0a	103.1±6.8a	0.51±0.02	2.14±0.16a	6.06±0.50
ANOVA							
F values	2.187	19.924	43.074	6.115	1.658	26.253	1.458
P values	NS	<0.001	<0.001	0.014	NS	<0.001	NS

Mean±standard error for each measure is given. In a column, different letters indicate significant differences among treatment according to the Duncan test ($P \leq 0.05$).

AM arbuscular mycorrhizal inoculum, *COW* composted olive-mill waste, *NS* not significant

Table 3 Soil properties in the rhizosphere soil of *T. articulata* grown in a heavy metal-polluted soil treated with composted olive-mill waste and inoculation with *G. mosseae* (n=5)

Treatment	pH	Electrical conductivity (dS m^{-1})	Total P (g kg^{-1})	Total K (g kg^{-1})	Total C (g kg^{-1})	Total N (g kg^{-1})	C/N ratio	TOC (g kg^{-1})	Aggregate stability (%)
AM	7.5±0.0	7.6±1.2	0.22±0.03	1.32±0.18	11.88±0.48c	0.45±0.08b	30	8.7±0.89b	9.2±0.84
AM + COW 1 %	7.5±0.1	4.5±0.7	0.14±0.02	1.39±0.10	17.00±1.02b	0.69±0.08b	26	10.8±0.31b	9.6±1,10
AM + COW 3 %	7.4±0.1	6.2±1.3	0.26±0.06	1.80±0.16	22.09±1.32a	1.23±0.19a	19	16.4±1.38a	10.2±0.37
ANOVA									
F values	0.871	2.118	2.048	2.964	26.074	10.145	–	17.120	0.341
P values	NS	NS	NS	NS	<0.001	0.003	–	<0.001	NS

Mean±standard error for each measure is given. In a column, different letters indicate significant differences among treatment according to the Duncan test ($P \leq 0.05$).

AM arbuscular mycorrhizal inoculum, *COW* composted olive-mill waste, *TOC* total organic carbon, *NS* not significant

exceeded that found in shoot tissues, the differences being particularly relevant for As and Pb (Table 7).

Discussion

Under the assayed conditions, the combined application of 3 % COW and mycorrhizal fungi inoculation in a Mediterranean soil contaminated by heavy metals increased the height and biomass accumulation of *T. articulata*. The fact that plants established without mycorrhizal inoculation or COW addition did not survive supports the premise that *T. articulata* in this multi contaminated soils need particular management practices to get established. Vegetation establishment on contaminated mine tailings is often difficult, the major constraints to plant establishment being metal toxicity, low nutrients availability, and poor soil aggregation (Chen et al. 2007). The composted olive-mill waste used in our study can be an organic matter and lignin source; it is also a source of K and P (Alburquerque et al. 2006), which can be related with an increase in plant growth as well as with the higher contents in foliar P. It is well known that the use of different amendments generates a better soil nutritional state (Valarini et al. 2009), including those that are contaminated by heavy metals (Medina et al. 2006). In this sense, in our study, total C, organic C, carbohydrates, and N in soil were increased with the treatments applied, which can increase nutrients' availability for plants (Medina et al. 2006). The use of amendments can influence positively the soil particles' aggregation (Carrasco et al. 2009), although their effectiveness depend on the content and nature of organic matter added and soil mineralogy (Paradelo et al. 2013). In our case, any of the doses assayed increased significantly the stability of soil aggregates, despite the addition of cementing agents such as polysaccharides contained in the COW (Alburquerque et al. 2006). This could be due to elevated soluble salt contents of this amendment (Fornes et al. 2009), which have a dispersing effect on soil aggregate stability (Paradelo et al. 2013). Organic amendments can also contribute to heavy metal immobilization through the mechanisms of adsorption, complexation, or co-precipitation (Santibañez et al. 2012). In our experiment, both doses of COW applied significantly decreased the Cr, Ni, and

Table 4 Mycelium density, AM colonization, glomalin, and carbohydrate contents in the rhizosphere of *T. articulata* grown in a heavy metal-polluted soil treated with composted olive-mill waste and inoculation with *G. mosseae* (n=5)

Treatment	Mycelium density (m mL^{-1})	Colonized root (%)	EE-GRSP (μg g^{-1})	Total CH (μg g^{-1})	Water-soluble CH (μg g^{-1})
AM	10.8±1.4	26.3±4.9	359±39 c	332±40 b	4±1 b
AM + COW 1 %	14.2±2.5	32.0±1.9	726±66 b	243±34 b	4±0 b
AM + COW 3 %	17.8±2.7	39.5±1.8	1010±98 a	536±71 a	13±1 a
ANOVA					
F values	2.369	2.869	20.546	60.946	30.665
P values	NS	NS	<0.001	<0.001	<0.001

Mean±standard error for each measure is given. In a column, different letters indicate significant differences among treatment according to the Duncan test ($P \leq 0.05$).

AM arbuscular mycorrhizal inoculum. *COW* composted olive-mill waste, *CH* carbohydrates, *EE-GRSP* easily extractable glomalin-related soil protein, *NS* not significant

Table 5 Enzymatic activities in the rhizosphere soil of *T. articulata* grown in a heavy metal-polluted soil treated with composted olive-mill waste and inoculation with *G. mosseae* (n=5)

Treatment	Dehydrogenase (μg g^{-1} INTF)	Urease (μmol NH_3 g^{-1} h^{-1})	Protease (μmol NH_3 g^{-1} h^{-1})	Phosphatase (μmol PNP g^{-1} h^{-1})	β-glucosidase (μmol PNP g^{-1} h^{-1})
AM	7.7±0.3 b	0.04±0.22b	0.13±0.03b	1.63±0.07b	0.02±0.01c
AM + COW 1 %	12.8±1.9 ab	0.19±0.05a	0.29±0.04a	1.84±0.10b	0.08±0.02b
AM + COW 3 %	13.8±2.2 a	0.27±0.03a	0.33±0.06a	2.19±0.09a	0.17±0.01a
ANOVA					
F values	7.704	9.069	5.239	10.402	33.634
P values	0.016	0.004	0.023	0.002	<0.001

Mean±standard error for each measure is given. In a column, different letters indicate significant differences among treatment according to the Duncan test ($P \leq 0.05$)

AM arbuscular mycorrhizal inoculum, *COW* composted olive-mill waste

Pb contents in shoot tissues, as well as Cr and As in root tissues. The lower metal concentrations in shoots of *T. articulata* plants amended with COW could be due to the higher biomass and the phytodilution effect. In agreement with the values given by Kabata-Pendias and Pendias (2001) As, Cu, Ni, Pb, and Zn concentrations measured in *T. articulate* shoots appeared to be in normal levels, regardless of whether the amendment is applied to soil. In contrast, shoot Cd and Cr concentrations could be considered excessive or toxic in vascular plants, but little information is available on the normal concentration for the species grown in non-contaminated soils. Both doses of COW did not decrease the toxic levels of shoot Cd, whereas the highest dose of COW decreased the uptake of Cr to shoot tissues until reaching normal levels. Cd concentrations above 0.5 mg kg^{-1} are considered as toxic for cattle (Chaney 1989), but *T. articulata* is unpalatable to herbivores and hence, it would not present a risk for metal accumulation in the food chain. On the other hand, the accumulation factors for each metal, defined as ratio between the concentration of metals in shoots and roots, were bellow 1, which indicates that *T. articulata* is a tolerant woody species and can be used in order to revegetate heavy metal-contaminated sites.

The contribution of AM fungi inoculation to alleviate heavy metal stress in plants has been demonstrated (Hildebrandt et al. 2007). Indeed, some studies describe that the AM mycorrhizal inoculation results in a reduction in the transplant-related mortality and an improvement in the growth of several plant species used for revegetation and phytoremediation programs (Carrasco et al. 2011) in heavy metal-contaminated soils. In this respect, metal retention in the rhizosphere soil of mycorrhizal plants has been attributed to a surface complexation on heavy metals with cysteine-containing ligands of fungal protein (Dehn and Schüepp 1989) including glycoproteins such as glomalin (González-Chávez et al. 2009; 2004) or other mechanisms which involve the action of extra-radical mycelium (Joner et al. 2000). In our study, the degree of root colonization in *T. articulata* was rather low (around 33 %), which contrasts with prior studies in the same species (Fernández et al. 2012) and another

Table 6 Total metal concentration in the rhizosphere soil of *T. articulata* grown in a heavy metal-polluted soil treated with composted olive-mill waste and inoculation with *G. mosseae* (n=5)

Treatment	As (μg g^{-1})	Cd (μg g^{-1})	Cr (μg g^{-1})	Cu (μg g^{-1})	Ni (μg g^{-1})	Pb (μg g^{-1})	Zn (μg g^{-1})
AM	422±26	33.3±1.7	45.6±2.8	207±10	29.1±1.7	7740±340	15500±990
AM + COW 1 %	356±26	32.0±0.8	43.3±0.8	192±5	28.2±0.4	7090±340	15000±870
AM + COW 3 %	408±46	33.9±1.2	45.7±2.3	209±4	30.1±0.8	7370±330	14600±780
ANOVA							
F values	1.058	0.552	0.380	1.815	0.755	0.928	0.263
P values	NS	NS	NS	NS	NS	NS	NS

Mean±standard error for each measure is given. In a column, different letters indicate significant differences among treatment according to the Duncan test ($P \leq 0.05$)

AM arbuscular mycorrhizal inoculum, *COW* composted olive-mill waste, *NS* not significant

Table 7 Metal concentration in the shoots and roots of *T. articulata* grown in a heavy metal-polluted soil treated with composted olive-mill waste and inoculation with *G. mosseae* ($n=5$)

Treatment	As ($\mu g\ g^{-1}$)		Cd ($\mu g\ g^{-1}$)		Cr ($\mu g\ g^{-1}$)		Cu ($\mu g\ g^{-1}$)		Ni ($\mu g\ g^{-1}$)		Pb ($\mu g\ g^{-1}$)		Zn ($\mu g\ g^{-1}$)	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
AM	<0.5	25.4±0.9 a	3.3±0.8	17.2±4.5	2.7±0.6 a	18.6±0.4 a	5.3±0.4	33.9±4.2	1.5±0.3 a	9.0±1.8	4.1±1.0 a	290±51	105±6	844±119
AM + COW 1 %	<0.5	15.0±1.3 b	3.2±0.5	15.8±3.3	1.7±0.1 ab	13.8±3.0 ab	6.1±0.4	29.6±3.6	0.9±0.1ab	6.7±1.6	1.6±0.2 b	196±29	148±16	661±47
AM + COW 3 %	<0.5	14.6±1.7 b	4.1±0.9	26.6±4.0	0.8±0.1 b	11.0±0.5 b	5.5±0.5	30.0±1.2	0.7±0.1 b	6.1±1.2	1.2±0.1 b	272±23	132±14	849±124
ANOVA														
F values	–	44.711	0.493	2.242	6.554	8.077	0.950	0.512	4.361	0.952	13.519	1.802	2.878	1.077
P values	–	<0.001	NS	NS	0.012	0.014	NS	NS	0.038	NS	0.003	NS	NS	NS

Mean±standard error for each measure is given. In a column, different letters indicate significant differences among treatment according to the Duncan test ($P\leq0.05$).
AM arbuscular mycorrhizal inoculum, *COW* composted olive-mill waste, *NS* not significant

species growing in metal-contaminated soils (Alguacil et al. 2011). The low values of root colonization and the absence of significant differences among treatments with regard to the density of AM mycelium can be explained by the high concentration of heavy metals present in the soil where the experiment was carried out. In this sense, some studies have shown that high metal concentration can inhibit AM spore germination (del Val et al. 1999), root colonization (Göhre and Paszkowski 2006), and extra-radical mycelium growth (del Val et al. 1999). Higher glomalin contents are usually observed after the addition of different organic sources, such as manure, crop stubbles, or compost (Valarini et al. 2009; Curaqueo et al. 2011) which is in agreement with our results; however, the quantity of glomalin extracted from the soil is typically related to the AM hyphal density (Lovelock et al. 2004). The AM fungus *G. mosseae* could be important for alleviating metal stress to plants grown in non-amended soil but not in conjunction with COW since the most of metals reached low levels and far from phytotoxic. Likewise, it appears that the AM fungus did not contribute to stimulate the growth of host plants grown in the amended soil.

The recovery of soil properties is a major goal in mine tailing phytostabilization programs. The improvement in soil quality can favor not only the establishment of the target plant species, but also favor the establishment of a spontaneous vegetal cover, thus contributing to the regeneration of these environments. Soil biochemical properties characterized by the enzyme activities are considered to be sensitive to pollution and have been proposed as indicators for measuring the degree of soil degradation (Trasar-Cepeda et al. 2000). In our study, the COW application in conjunction with AM fungi inoculation resulted in an improvement on dehydrogenase, alkaline phosphatase, β-glucosidase, urease, and protease-BAA activities. In general, enzymatic activities are closely related with the nutrient cycles and transformation (Gianfreda et al. 2005). The presence of heavy metals in the soil may depress the biochemical processes by affecting enzyme activities (Khan et al. 2007). Heavy metals may inhibit enzyme activities by masking catalytically active groups, having denaturing effects on the conformation of proteins, or competing with the metal ions involved in the formation of enzyme–substrate complexes (Gianfreda et al. 2005). The addition of organic amendments such as crop residues or compost can enhance diverse enzyme activities (García-Gil et al. 2000), even in contaminated soils (Fernández et al. 2012). In our study, the COW application mitigated the inhibition effects of heavy metals on enzyme activities, and this positive effect was more evident with the highest dose applied. Also, the highest COW dose applied significantly improved the soil carbohydrates content, which can act as an easily available nutrient source for soil microorganisms (Carrasco et al. 2011) and aid to the recovery of the soil biological productivity.

Conclusions

Our results evidence a beneficial effect of the amendment with composted olive waste in combination with AM fungi on growth of *T. articulata*, as well as a clear improvement in soil properties in the phytostabilization of a mine tailing with high levels of heavy metals and As. The olive-mill waste compost aided *G. mosseae*-inoculated *T. articulata* plants to thrive in contaminated soil, mainly through an improvement in both nutrients uptake, mainly P and soil microbial function. The improvement in growth of mycorrhizal *T. articulata* plants produced by the amendment does not seem to be related to alleviation of metal stress in plant performance since most of them reached low levels and are far from phytotoxic. The combined use of amendment plus AM fungi could be a feasible strategy not only for mine tailing regeneration, but also for the disposal of the olive-mill wastes. Caution is needed in extrapolating these results to the field, as plant performance might not be as great as under controlled conditions despite being a woody species adapted to semiarid climate conditions.

Acknowledgments G. Curaqueo thanks Fundación Carolina (Spain) and Universidad de La Frontera (Chile) for supporting his Postdoctoral stay at CEBAS-CSIC, Murcia, Spain. M. Schoebitz would like to thank the National Commission for Scientific and Technological Research of Chile (CONICYT) for the postdoctoral fellowship.

References

Adriano DC, Wenzel WW, Vangronsveld J, Bolan NS (2004) Role of assisted natural remediation in environmental cleanup. Geoderma 122:121–142

Alburquerque JA, Gonzálvez J, García D, Cegarra J (2006) Composting of a solid olive-mill by-product ("alperujo") and the potential of the resulting compost for cultivating pepper under commercial conditions. Waste Manag 26:620–626

Alburquerque JA, de la Fuente C, Bernal MP (2011) Improvement of soil quality after "alperujo" compost application to two contaminated soils characterised by differing heavy metal solubility. J Environ Manag 92:733–741

Alguacil MM, Torrecillas E, Caravaca F, Fernández DA, Azcón R, Roldán A (2011) The application of an organic amendment modifies the arbuscular mycorrhizal fungal communities colonizing native seedlings grown in a heavy-metal-polluted soil. Soil Biol Biochem 43:1498–1508

Azcón R, Medina A, Roldán A, Biró B, Vivas A (2009) Significance of treated agrowaste residue and autochthonous inoculates (arbuscular mycorrhizal fungi and *Bacillus cereus*) on bacterial community structure and phytoextraction to remediate soil contaminated with heavy metals. Chemosphere 75:327–334

Barea JM, Palenzuela J, Cornejo P, Sánchez-Castro I, Navarro-Fernández C, Lopéz-García A, Estrada B, Azcón R, Ferrol N, Azcón-Aguilar C (2011) Ecological and functional roles of mycorrhizas in semi-arid ecosystems of Southeast Spain. J Arid Environ 75:1292–1301

Brink RH, Dubar P, Lynch DL (1960) Measurement of carbohydrates in soil hydrolysates with anthrone. Soil Sci 89:157–166

Carrasco L, Azcón R, Kohler J, Roldán A, Caravaca F (2011) Comparative effects of native filamentous and arbuscular mycorrhizal fungi in the establishment of an autochthonous, leguminous shrub growing in a metal-contaminated soil. Sci Total Environ 409:1205–1209

Carrasco L, Caravaca F, Azcón R, Kohler J, Roldán A (2009) Addition of microbially-treated sugar beet residue and a native bacterium increases structural stability in heavy metal-contaminated Mediterranean soils. Sci Total Environ 407:5448–5454

Chaney RL (1989) Toxic element accumulation in soils and crops: protecting soil fertility and agricultural food-chains. In: Bar-Yosef B, Barrow NJ, Goldshmid J (eds) Inorganic Contaminants in the Vadose Zone. Springer, Berlin, pp 140–158

Chen BD, Zhu YG, Duan J, Xiao XY, Smith SE (2007) Effects of the arbuscular mycorrhizal fungus *Glomus mosseae* on growth and metal uptake by four plant species in copper mine tailings. Environ Pollut 147:374–380

Ciccu R, Ghiani M, Serci A, Fadda S, Peretti R, Zucca A (2003) Heavy metal immobilization in the mining-contaminated soils using various industrial wastes. Miner Eng 16:187–192

Conesa HM, Faz A, Arnaldos R (2006) Heavy metal accumulation and tolerance in plants from mine tailings of the semiarid Cartagena–La Unión mining district (SE Spain). Sci Total Environ 366:1–11

Conesa HM, Faz A, Arnaldos R (2007) Initial studies for the phytostabilization of a mine tailing from the Cartagena-La Union Mining District (SE Spain). Chemosphere 66:38–44

Córdova S, Neaman A, González I, Ginocchio R, Fine P (2011) The effect of lime and compost amendments on the potential for the revegetation of metal-polluted, acidic soils. Geoderma 166:135–144

Curaqueo G, Barea JM, Acevedo E, Rubio R, Cornejo P, Borie F (2011) Effects of different tillage system on arbuscular mycorrhizal fungal propagules and physical properties in a Mediterranean agroecosystem in central Chile. Soil Till Res 113:11–18

Da Silva S, Siqueira J, Fonsêca C (2006) Mycorrhizal fungi influence on brachiariagrass growth and heavy metal extraction in a contaminated soil. Pesqui Agropecu Bras 41:1749–1757

Dehn B, Schüepp H (1989) Influence of VA mycorrhizae on the uptake and distribution of heavy metals in plants. Agr Ecosys Environ 29: 79–83

Del Val C, Barea JM, Azcón-Aguilar C (1999) Assessing the tolerance to heavy metals of arbuscular mycorrhizal fungi isolated from sewage sludge-contaminated soils. Appl Soil Ecol 11:261–269

Fernández DA, Roldán A, Azcón R, Caravaca F, Bååth E (2012) Effects of water stress, organic amendment and mycorrhizal inoculation on soil microbial community structure and activity during the establishment of two heavy metal-tolerant native plant species. Microbial Ecol 63:794–803

Fornes F, García-de-la-Fuente R, Belda RM, Abad M (2009) 'Alperujo' compost amendment of contaminated calcareous and acidic soils: effects on growth and trace element uptake by five *Brassica* species. Bioresour Technol 100:3982–3990

García C, Hernández MT, Costa F (1997) Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun Soil Sci Plan 28:123–134

García-Gil JC, Plaza C, Soler-Rovira P, Polo A (2000) Long-term effects of municipal solid waste compost application on soil enzyme activities and microbial biomass. Soil Biol Biochem 32:1907–1913

Gianfreda L, Rao MA, Piotrowska A, Palumbo G, Colombo C (2005) Soil enzyme activities as affected by anthropogenic alterations: intensive agricultural practices and organic pollution. Sci Total Environ 341:265–279

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytol 84:489–500

Göhre V, Paszkowski U (2006) Contribution of the arbuscular mycorrhizal symbiosis to heavy metal phytoremediation. Planta 223:1115–1122

González-Chávez MC, Carrillo-González R, Gutiérrez-Castorena MC (2009) Natural attenuation in a slag heap contaminated with cadmium: the role of plants and arbuscular mycorrhizal fungi. J Hazard Mater 161:1288–1298

González-Chávez MC, Carrillo-González R, Wright SF, Nichols KA (2004) The role of glomalin, a protein produced by arbuscular mycorrhizal fungi, in sequestering potentially toxic elements. Environ Pollut 130:317–323

Gosling P, Hodge A, Goodlass G, Bending GD (2006) Arbuscular mycorrhizal fungi and organic farming. Agr Ecosyst Environ 113:17–35

Hildebrandt U, Regvar M, Bothe H (2007) Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry 68:139–146

Houben D, Pircar J, Sonnet P (2012) Heavy metal immobilization by cost-effective amendments in a contaminated soil: effects on metal leaching and phytoavailability. J Geochem Explor 123:87–94

Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea JM (2003) The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils 37:1–16

Joner E, Briones R, Leyval C (2000) Metal-binding capacity of arbuscular mycorrhizal mycelium. Plant Soil 226:227–234

Kabata-Pendias A, Pendias H (2001) Trace elements in soils and plants, 3rd edn. CRC Press, Boca Raton, Florida

Kabas S, Acosta JA, Zornoza R, Faz Cano A, Carmona DM, Martinez-Martinez S (2011) Integration of landscape reclamation and design in a mine tailing in Cartagena-La Unión, SE Spain. Int J Energy Environ 5:301–308

Khan S, Cao Q, Hesham AEL, Xia Y, He JZ (2007) Soil enzymatic activities and microbial community structure with different application rates of Cd and Pb. J Environ Sci 19:834–840

Lax A, Diaz E, Castillo V, Albadejo J (1994) Reclamation of physical and chemical properties of a salinized soil by organic amendment. Arid Soil Res Rehab 8:9–17

Lovelock CE, Wright SF, Nichols KA (2004) Using glomalin as an indicator for arbuscular mycorrhizal hyphal growth: an example from a tropical rain forest soil. Soil Biol Biochem 36:1009–1012

Martínez-Fernández D, Walker DJ (2012) The effects of soil amendments on the growth of *Atriplex halimus* and *Bituminaria bituminosa* in heavy metal-contaminated soils. Water Air Soil Poll 223:63–72

Masciandaro G, Ceccanti B, Garcia C (1994) Anaerobic digestion of straw and pig waste water. II. Optimization of the process. Agrochimica 3:195–203

Medina A, Vassileva M, Barea JM, Azcón R (2006) The growth-enhancement of clover by *Aspergillus*-treated sugar beet waste and *Glomus mosseae* inoculation in Zn contaminated soil. Appl Soil Ecol 33:87–98

Meier S, Borie F, Bolan N, Cornejo P (2012) Phytoremediation of metal-polluted soils by arbuscular mycorrhizal fungi. Crit Rev Env Sci Tec 42:741–775

Mendez MO, Maier RM (2008) Phytoremediation of mine tailings in temperate and arid environments. Rev Environ Sci Biotechnol 7:47–59

Morais S, Garcia e Costa F, Pereira ML (2012) Heavy metals and human health. In: Oosthuizen J (ed) Environmental Health-Emerging Issues and Practice. InTech, Rijeka, Croatia, pp 227–246

Murphy J, Riley JP (1962) A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta 27: 31–36

Nannipieri P, Ceccanti B, Cervelli S, Matarese E (1980) Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil. Soil Sci Soc Am J 44:1011–1016

Naseby DC, Lynch JM (1997) Rhizosphere soil enzymes as indicators of perturbation caused by a genetically modified strain of *Pseudomonas fluorescens* on wheat seed. Soil Biol Biochem 29: 1353–1362

Norland MR, Veith DL (1995) Revegetation of coarse taconite iron ore tailing using municipal waste compost. J Harzard Mater 41:123–134

Oehl F, Sieverding E, Mäder P, Dubois D, Ineichen K, Boller T, Wiemken A (2004) Impact of long-term conventional and organic farming on the diversity of arbuscular mycorrhizal fungi. Oecologia 138:574–583

Oen IS, Fernández JC, Manteca JI (1975) The lead–zinc and associated ores of La Unión, Sierra de Cartagena, Spain. Econ Geol 70:1259–1278

Pagliai M, Vignozzi N, Pellegrini S (2004) Soil structure and the effect of management practices. Soil Till Res 79:131–143

Paradelo R, van Oort F, Chenu C (2013) Water-dispersible clay in bare fallow soils after 80 years of continuous fertilizer addition. Geoderma 200–201:40–44

Pardo T, Martínez-Fernández D, Clemente R, Walker DJ, Bernal MP (2013) The use of compost to promote the plant vegetation cover in a trace elements-contaminated soil. Environ Sci Pollut R. doi:10.1007/s11356-013-1988-z

Rubio R, Borie F, Schalchli C, Castillo C, Azcón R (2003) Occurrence and effect of arbuscular mycorrhizal propagules in wheat as affected by source and amount of phosphorus fertilizer and fungal inoculation. Appl Soil Ecol 23:245–255

Santibañez C, de la Fuente LM, Bustamante E, Silva S, León-Lobos P, Ginocchio R (2012) Potential use of organic- and hard-rock mine wastes on aided phytostabilization of large-scale mine tailings under semiarid Mediterranean climatic conditions: short-term field study. Appl Environ Soil Sci. doi:10.1155/2012/895817

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic, Amsterdam

Tabatabai MA, Bremmer JM (1969) Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol Biochem 1:301–307

Tabatabai MA (1982) Soil enzymes. In: Page AL, Miller EM, Keeney DR (eds) Methods of soil analysis. ASA and SSSA, Madison, pp 501–538

Tordoff GM, Baker AJM, Willis AJ (2000) Current approaches to the revegetation and reclamation of metalliferous mine wastes. Chemosphere 41:219–228

Trasar-Cepeda C, Leirós MC, Seoane S, Gil-Sotres F (2000) Limitations of soil enzymes as indicators of soil pollution. Soil Biol Biochem 32: 1867–1875

Valarini PJ, Curaqueo G, Seguel A, Manzano K, Rubio R, Cornejo P, Borie F (2009) Effect of compost application on some properties of a volcanic soil from central south Chile. Chil J Agri Res 69:416–425

Wong MH (2003) Ecological restoration of mine degraded soils, with emphasis on metal contaminated soils. Chemosphere 50: 775–780

Wright SF, Starr JL, Paltineanu IC (1999) Changes in aggregate stability and concentration of glomalin during tillage management transition. Soil Sci Soc Am J 63:1825–1829

Wright SF, Upadhyaya A (1998) A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. Plant Soil 198:97–107