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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

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Abstract Our aim was to examine the effect of water stress on plant growth and development of two native plant species (*Tetraclinis articulata* and *Crithmum maritimum*) and on microbial community composition and activity in the rhizosphere soil, following the addition of an organic amendment, namely sugar beet residue (SBR), and/or the inoculation with an arbuscular mycorrhizal (AM) fungus, namely *Glomus mosseae*, in a non-sterile heavy metal-polluted soil. The AM inoculation did not have any significant effect on plant growth of both species. In *T. articulata*, SBR increased shoot growth, foliar P, total phospholipid fatty acids (PLFA), fungi-related PLFA, AM fungi-related neutral lipid fatty acid, bacterial gram-positive/gram-negative PLFA ratio and the β-glucosidase and dehydrogenase activities. SBR and AM inoculation increased phosphatase activity in *T. articulata* plants grown under drought conditions. In both plants, there was a synergistic effect between AM inoculation and SBR on mycorrhizal colonisation under drought conditions. In *C. maritimum*, the increase produced by the SBR on total amounts of PLFA, bacterial gram-positive-related PLFA and bacterial gram-negative-related PLFA was considerably higher under drought conditions. Our results suggest that the effectiveness of the amendment with regard to stimulating microbial communities and plant growth was largely limited by drought, particularly for plant species with a low degree of mycorrhizal colonisation.

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Introduction

In the last 200 years, the area surrounding La Unión, in the SE of Spain, has been developed as an important mining zone. However, heavy metals have been released into the area as a result of mining activities, since before Roman times. In addition to this, the mining district has a semiarid Mediterranean climate with high temperature, high irradiance and low irregular rainfall (rarely above 350 mm), so that plants are subjected to a high degree of environmental stress, especially during the summer months. The high heavy metal concentrations and the arid climate are obstacles to the establishment of vegetation, which could prevent soil erosion and the subsequent spread of toxic metals. Selection of appropriate plant species which can establish, grow and colonise metal-contaminated soils is thus important for successful remediation of these sites. Drought-resistant and metal-tolerant native plant species should be used in order to achieve self-sustainable vegetation on semiarid, contaminated lands [8].

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts which are widely distributed among higher plant species, being the dominant beneficial fungi colonising the roots of most species [38]. The AMF are integral,

functional parts of plant roots, enhancing nutrient availability and plant tolerance to biotic and abiotic stresses, including water stress and metal toxicity [3, 18]. In addition, the AM fungi may indirectly influence plant growth by changing the composition and activity of microbial communities in the mycorrhizosphere, which play a key role in the functioning of soil, in nutrient cycling and structural formation [21]. Mycorrhiza formation can affect the microbial population in the rhizosphere directly or indirectly through changes in composition and quantity of root exudates, or through fungal exudates. Changes in composition and activity of bacterial communities by introduced AM fungi have been previously described [2]. In contrast, the impact of AM inoculation on the structure of bacterial communities in natural soil with high-grade metal toxicity is scarce [5].

There is growing evidence that soil enzyme activity may have a potential role as early and sensitive indicators of soil ecological stress and restoration [29, 30]. Soil enzyme activities also have been successfully used as early and sensitive indicators for reflecting the degree of quality reached by a soil in the rehabilitation process [6]. This approach provides a comprehensive view of the impact of an inoculant on the functioning of the soil ecosystem. However, there are relatively few studies regarding the use of such parameters as a method to monitoring the impact of mycorrhizal inoculation for restoration of mine-polluted soils.

Sugar beet residue (SBR), a lignocellulosic material, is obtained as a by-product after the extraction of sugar by industrial procedures. The fermentation of SBR supplemented with rock phosphate by *Aspergillus niger* provides an organic amendment rich in polysaccharide compounds and available P [5, 28]. The fermented *A. niger* sugar beet waste increases growth and P uptake by the AM mycelium in soil whereas nonfermented SBR has a strong inhibitory effect due to its component ferulic acid [27]. In addition to this, applying fermented SBR to contaminated soils adds value to a biological material which otherwise would represent a waste problem for the sugar industry.

Both AM inoculation and organic matter addition may change the composition of the soil microbial community [5]. Analysing the response of microorganisms to different treatments is important because soil microbiota respond quickly to environmental changes, so they are expected to be efficient bioindicators of soil conditions [4]. In order to monitor those changes, phospholipid fatty acid (PLFA) and neutral lipid fatty acid (NLFA) profilings have been shown to be reliable bioindicators [9].

The main aim of this study was to evaluate the effects of the addition of an organic amendment (SBR) and/or the inoculation with an AM fungus on the growth and development of two native plant species (*Tetraclinis articulata* and *Crithmum maritimum*) in a non-sterile heavy metal-polluted soil under drought conditions. We also studied whether the effects of phytoremediation treatments were linked to changes in the microbial community composition and activity of the rhizosphere soil. For this experiment, a *Glomus mosseae* strain isolated from the mine tailings was selected. The results obtained will determine the usefulness of SBR as a soil amendment for phytoremediation and recovery purposes of polluted soils in semiarid environments.

Materials and Methods

Study Site

The study area was located in the La Unión mine district (southeast Spain). The terrain is low lying (<400 m), but with steep slopes (20–30%) because of its proximity to the coast. The climate is semiarid Mediterranean with an annual rainfall around 250–300 mm and a mean annual temperature of 17.5°C; the potential evapotranspiration reaches 1,000 mm year^{-1}. This district has been an important mining nucleus for more than 2,500 years. The ore deposits have iron, lead and zinc as the main metal components. Iron is present in oxides, hydroxides, sulphides, sulphates, carbonates and silicates; lead and zinc occur in galena, sphalerite, carbonates, sulphates and lead- or zinc-bearing (manganese, iron) oxides [8]. In this area, a neutral mine tailing called "El Gorguel" (U.T.M. X687480 Y4162800 Z135; length, 200–300 m; width, 95 m; height, 25 m; volume, 750,000 m^3) was selected. Three separate soil samples were taken from the tailing. Each soil sample consisted of a mixture of six subsamples randomly taken from the top 20 cm of soil. The physico-chemical characteristics of the mine tailing are shown in Table 1.

Materials

SBR was dried at 60°C and then sieved to pass a 2-mm pore mesh. Portions of 15 g of SBR were mixed with 40 mL of Czapek solution (agar, 15.0 g L^{-1}; dipotassium hydrogen phosphate, 1.0 g L^{-1}; iron (II) sulphate heptahydrate, 0.01 g L^{-1}; potassium chloride, 0.5 g L^{-1}; magnesium sulphate heptahydrate, 0.5 g L^{-1}; sodium nitrate, 3.0 g L^{-1}; sucrose, 30.0 g L^{-1}; pH=7.3) for static fermentation in 250-mL Erlenmeyer flasks. Rock phosphate (Morocco fluorapatite, 12.8% P, 1-mm mesh) was added at a rate of 0.75 g per flask. Media were sterilised by autoclaving at 120°C for 30 min. A spore suspension of *A. niger* NB2 (1.2×10^7) was spread carefully over the surface of the media. The mixture was allowed to ferment at 30°C for 20 days without shaking. The characteristics of

Table 1 Physico-chemical properties of the polluted soil

pH	7.7±0.1[a]
EC (mS cm^{-1})	1.3±0.1
Al (g kg^{-1})	14.5±0.3
Ca (g kg^{-1})	7.9±0.6
Cd (mg kg^{-1})	37±1
Cr (mg kg^{-1})	91±3
Cu (mg kg^{-1})	163±6
Fe (g kg^{-1})	190.3±5.1
K (mg kg^{-1})	560±26
Mg (g kg^{-1})	11.9±0.4
Mn (g kg^{-1})	5.9±0.2
Na (mg kg^{-1})	273±5
Ni (mg kg^{-1})	15±0
P (g kg^{-1})	6.4±0.2
P available (mg kg^{-1})	7±1
Pb (g kg^{-1})	6.9±0.0
S (g kg^{-1})	12.7±0.3
Zn (g kg^{-1})	12.0±0.3

[a] Mean ± standard error

EC electrical conductivity

the SBR after fermentation were: pH 5.3; total P, 224 μg mL^{-1}; total N, 1.2%; cellulose, 11.3%; hemicellulose, 3.1%; lignin, 4.1% and reducing sugar, 0.25 g L^{-1}.

The mycorrhizal inoculum, originating from the experimental area, was a *G. mosseae* strain being the most abundant AM spore in this soil [5]. The AM inoculum consisted of a mixture of rhizospheric soil from trap cultures (*Sorghum bicolor*) containing spores, hyphae and mycorrhizal root fragments.

The plants used were *T. articulata* L. and *C. maritimum* L. *T. articulata* is an evergreen coniferous tree in the family Cupressaceae. It is native to the experimental area and grows near the mine tailings. *C. maritimum* is a shrub in the family Apiaceae which grows near the coast. Plants were grown in a nursery with peat as substrate for 10 months prior to experimental procedures. At planting, *T. articulata* was 45.3±1.4 cm high, with a shoot dry weight of 5.76± 0.93 g (n=3), while *C. maritimum* was 11.7±0.5 cm high, with a shoot dry weight of 0.28±0.02 g (n=3).

Experimental Design

The experiment was a mesocosm assay, conducted as a complete randomised factorial design with three factors. The first factor had two levels: non-addition or addition of fermented sugar beet residue; the second had two levels: non-inoculation or inoculation with *G. mosseae* and the third one had two levels: well watered and water stress. Five replicates per treatment were used, making a total of 40 pots per plant species.

One kilogram of air-dried soil was placed in 1,500-mL pots. *T. articulata* and *C. maritimum* plants were then transplanted to the pots. When appropriate, 12 g of sugar beet residue was mixed with the soil (1.2% *w*/*w*). The arbuscular mycorrhizal inoculum was applied at a rate of 5% (*v*/*v*). The same amount of the autoclaved inoculum was added to non-mycorrhizal plants, supplemented with a filtrate (Whatman no. 1 paper) of the culture to provide the microbial populations accompanying the mycorrhizal fungi. The experiment was carried out in the greenhouse with an average maximum temperature of 22°C. Plants were well watered with decalcified water without any fertiliser treatment. Five months after planting, half of the plants were separated and deprived of watering for a whole month. Six months after planting, plants were harvested.

Plant Analyses

Fresh and dry mass of shoots and roots (105°C, 5 h) were recorded. Leaves were ground after drying. About 0.6 g of ground dry leaves was burnt in a muffle (480°C, 15 h). When cool, ashes were dissolved in HNO_3 (0.6 N) and filtered through an Albert® 145 ashless filter paper. This acid extract was then used to quantify phosphorus in leaves using an ICP-MS (Thermo Electron Corporation Mod. IRIS Intrepid II XDL).

Roots were subsampled in three 2-cm cross sections of the upper, middle and lower root system. To assess mycorrhizal colonisation, roots were cleared with 10% KOH and stained with 0.05% trypan blue [32]. Roots from *T. articulata* were too dark and needed an initial step of clearing with 3% *v*/*v* H_2O_2 (10 vol.). The percentage of root length colonised by AM fungi was calculated by the gridline intersect method [17]. Positive counts for AM colonisation included the presence of vesicles or arbuscules or typical mycelium within the roots.

Soil Analyses

To collect the rhizosphere soil, the root system with rhizosphere soil adhered was introduced into a plastic bag, shook and separated the rhizosphere soil from the root system. Rhizosphere soil samples sieved to <2 mm were divided into two subsamples: one subsample was stored at 2°C for microbiological analysis, and another subsample was allowed to dry at room temperature for physical–chemical analysis.

Total metal contents were determined by nitric–perchloric digestion: 1 g of crushed sample was placed in a Kjeldahl flask, and 10 mL of concentrated HNO_3 plus 10 mL of concentrated $HClO_4$ was added. The mixture was

heated at 210°C for 90 min and then left to cool down at room temperature. When cool, the content of the tubes was filtered through an Albert® 145 ashless filter paper, and the volume completed at 50 mL by washing the Kjeldahl flasks with 0.5 N HCl several times. All metals were quantified using an inductively coupled plasma mass spectrometry (ICP-MS). The precision and accuracy of this method were tested by analysing (five replicates) the CTA-VTL-2 [12] and CRM027-050 (Resource Technology Corporation, USA) certified materials, corresponding to Virginia tobacco leaves and a soil, respectively.

Dehydrogenase activity was determined according to García et al. [15]. For this, 1 g of soil at 60% of its field capacity was exposed to 0.2 mL of 0.4% 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride in distilled water for 20 h at 22°C in the dark. The iodonitrotetrazolium formazan (INTF) formed was extracted with 10 mL of methanol by shaking vigorously for 1 min and filtering through Whatman no. 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

Acid phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (0.115 M) as substrate. Two millilitres of 0.5 M sodium acetate buffer at pH 5.5 using acetic acid [30] 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 was centrifuged at 4,000 rpm for 5 min. The *p*-nitrophenol (PNP) formed was determined by spectrophotometry at 398 nm. Controls were made in the same way, although the substrate was added before the $CaCl_2$ and NaOH.

β-Glucosidase was determined using *p*-nitrophenyl-β-D-glucopyranoside (0.05 M) as substrate. This assay is based on the release and detection of PNP. Two millilitres of 0.1 M maleate buffer 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 trishydroxymethyl aminomethane according to Tabatabai [39]. The amount of PNP was determined by spectrophotometry at 398 nm.

PLFA extraction and analysis was carried out as described in Frostegård et al. [14]. Briefly, 1.0 g (fresh weight) of soil was extracted with a chloroform–methanol–citrate buffer (1:2:0.8). The resulting lipid material was fractionated into neutral lipids, glycolipids and phospholipids on a silica column by elution with chloroform, acetone and methanol, respectively. Neutral lipids and phospholipids were subjected to mild alkaline methanolysis, and the fatty acid methyl esters were separated by gas chromatography (flame ionization detector) using a 30-m HP-5 (phenylmethyl silicone) capillary column. Hydrogen was used as a carrier gas. Peak areas were quantified by adding methyl nonadecanoate fatty acid (19:0) as an internal standard.

Statistical Analysis

Percentage colonisation was arcsin transformed, and the other parameters were log transformed to achieve normality. The effects of amendment addition, mycorrhizal inoculation, water stress and their interactions on measured variables were tested by a three-factor ANOVA. Significant interaction was a posteriori explored with the Tukey honestly significant difference (HSD) test, calculated at $P<0.05$. All statistical analyses were performed using SPSS 17.0 for Windows.

Results

Plant Parameters

The addition of SBR significantly improved shoot growth of *T. articulata*, while water stress decreased it (Table 2; $P=0.035$ and $P=0.046$, respectively). The results of ANOVA indicated that the addition of the AM fungus had no effect on *T. articulata* shoot growth. Root growth was not affected significantly by any factor. There was a significant water stress × mycorrhizal inoculation interaction for shoot water content (S×M interaction, $P=0.018$); it was enhanced by *G. mosseae* in stressed plants but was not affected by the mycorrhizal inoculation in non-stressed plants. Only the addition of SBR significantly increased the content of foliar P by 75% in amended plants as compared to non-amended plants (Table 2, $P<0.001$).

For *C. maritimum* plants, neither the addition of SBR nor the inoculation with the AM fungus had a significant effect on shoot dry weight based on ANOVA (Table 2), while water stress decreased it. The shoot water content was also affected strongly ($P<0.001$) by the water stress, decreasing by up to 12% with respect to well-watered plants. Root growth and foliar P were not affected significantly by any factor. There was a significant water stress × SBR amendment × AM inoculation interaction (Table 2, $P=0.015$) for mycorrhizal colonisation of *T. articulata* roots such that in water-stressed plants, the greatest colonisation was found when both residue and AM inoculum were present.

In *C. maritimum*, root colonisation was generally lower than in *T. articulata*. Also, there was a significant interaction among all three experimental factors on mycorrhizal colonisation (Table 2, $P=0.043$). The existence of a synergistic effect between AMF inoculation and SBR amendment under drought conditions was confirmed by the Tukey HSD test ($P=0.002$).

Rhizospheric Microbial Community Parameters

For *T. articulata* plants, the addition of the SBR promoted the rhizospheric populations of all the microbial groups

Table 2 Effect of the addition of sugar beet residue and inoculation with *G. mosseae* on shoot biomass, shoot water content, foliar phosphorus and mycorrhizal colonisation of *T. articulata* and *C. maritimum* roots grown in a heavy metal-polluted soil under two different levels of watering

	Shoot dry biomass (g dry weight)		Shoot water content (%)		Foliar P (mg plant^{-1})		Colonisation (%)	
	Ta	Cm	Ta	Cm	Ta	Cm	Ta	Cm
Well watered								
Control	12.2	5.6	53.4	86.0	8	9	54.3	28.3
SBR	14.7	7.0	51.0	84.3	14	11	67.3	50.3
AMF	10.1	5.6	51.4	85.1	8	12	52.3	45.3
SBR + AMF	19.4	5.5	48.3	85.4	15	13	51.0	47.0
Water stressed								
Control	11.4	4.6	37.7	69.1	9	9	51.3	26.3
SBR	10.8	4.9	38.7	76.6	13	10	53.0	26.0
AMF	9.8	3.3	50.8	77.7	6	6	50.3	36.0
SBR + AMF	12.0	3.2	38.2	74.9	11	7	68.0	40.7
s.e.d.	2.1	0.6	3.3	1.9	2.4	1.2	5.6	6.1
ANOVA, *P* values								
Water stress (*S*)	0.046	0.018	<0.001	<0.001	NS	NS	NS	0.002
Amendment (*A*)	0.035	NS	0.019	NS	<0.001	NS	0.014	0.027
Mycorrhiza (*M*)	NS	NS	NS	NS	NS	NS	NS	0.005
S×*A*	NS	NS	NS	NS	NS	NS	NS	NS
S×*M*	NS	NS	0.018	NS	NS	NS	0.010	NS
A×*M*	NS	NS	0.041	NS	NS	NS	NS	NS
S×*A*×*M*	NS	NS	NS	NS	NS	NS	0.015	0.043

Significance of effects of water stress, amendment and AM inoculation on the measured variables are also shown

SBR sugar beet residue, *AMF* arbuscular mycorrhizal fungi, *s.e.d.* standard error of difference between two means, *NS* not significant at $P>0.05$

studied (Tables 3 and 4), increasing total PLFA ($P<0.001$), fungi-related PLFA ($P=0.001$) and AM fungi-related NLFA ($P=0.008$). SBR addition also enhanced the gram-positive-related PLFA ($P<0.001$) and gram-negative-related PLFA ($P<0.001$), particularly the gram-positive-related ones. There was a significant effect of the watering regime on the amount of bacterial gram-positive-related PLFA, which decreased due to drought stress.

In *C. maritimum* plants, there was a significant interaction between the addition of SBR and the water regime, indicating that the effect of SBR amendment on total amounts of PLFA, bacterial gram-positive-related PLFA and bacterial gram-negative-related PLFA was considerably higher under drought conditions than under watering conditions. The main effect of amendment was significant, enhancing fungi-related PLFA ($P<0.001$) and AM fungi-related NLFA ($P=0.018$). Also, SBR addition enhanced the gram-positive/gram-negative ratio ($P=0.019$), producing a higher increase in the gram-positive component than in the gram-negative component. According to Tukey HSD test ($P<0.001$), the SBR was effective in increasing the amount of gram-negative-related PLFA, irrespectively of the presence of the AM fungus.

Soil Enzymatic Activities

There was a significant interaction among water stress × SBR amendment × AM inoculation on phosphatase activity in the rhizospheric soil from *T. articulata* (Table 5, $P=0.020$). The post hoc test showed that the addition of amendment and AM inoculation increased phosphatase activity, but this effect was significant only in plants grown under drought conditions. The combination of SBR amendment and AM inoculation had a similar effect to the AM inoculation alone. The ANOVA results revealed significant increases in β-glucosidase ($P<0.001$) and dehydrogenase ($P<0.001$) activities with the addition of the SBR (Table 5). No significant interaction effects between water regime and mycorrhizal inoculation were confirmed according to Tukey HSD test.

In *C. maritimum*, there is a significant S × M interaction for the acid phosphatase activity (Table 5, $P=0.014$). According to the post hoc test, stress water produced a significant reduction in the soil phosphatase activity of plants inoculated with the AM fungus. Only the addition of the SBR caused significant increases in β-glucosidase ($P<0.001$) and dehydrogenase ($P<0.001$) activities (Table 5).

Table 3 Effect of the addition of sugar beet residue and inoculation with *G. mosseae* on total amounts of fatty acids, relative abundance of bacterial phospholipid fatty acids biomarkers and gram-positive/gram-negative ratio in the rhizosphere soil of *T. articulata* and *C. maritimum* grown in a heavy metal-polluted soil under two different levels of watering

	Total PLFA (nmol g^{-1})		Gram-positive PLFA (nmol g^{-1})		Gram-negative PLFA (nmol g^{-1})		Gram positive/gram negative	
	Ta	Cm	Ta	Cm	Ta	Cm	Ta	Cm
Well watered								
Control	7.2	11.4	1.2	1.6	1.1	1.2	1.1	1.3
SBR	20.6	24.8	4.2	5.0	3.0	3.2	1.4	1.5
AMF	6.8	8.0	1.0	1.3	0.5	1.3	2.0	1.0
SBR + AMF	21.4	15.0	4.1	3.2	2.7	2.4	1.5	1.3
Water stressed								
Control	6.5	3.8	0.9	0.5	0.8	0.6	1.0	0.8
SBR	20.5	16.6	3.6	3.8	2.6	3.0	1.7	1.3
AMF	4.0	5.2	0.4	0.8	0.5	0.8	0.9	1.0
SBR + AMF	15.6	16.4	2.8	3.6	2.4	2.7	1.2	1.3
s.e.d.	5.6	5.0	1.2	1.2	0.8	0.8	0.2	0.2
ANOVA, *P* values								
Water stress (*S*)	NS	0.007	0.024	0.008	NS	0.001	NS	NS
Amendment (*A*)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.019
Mycorrhiza (*M*)	NS	NS	NS	NS	0.025	NS	NS	NS
S×*A*	NS	0.044	NS	0.023	NS	0.001	NS	NS
S×*M*	NS	NS	NS	NS	NS	NS	NS	NS
A×*M*	NS	NS	NS	NS	NS	0.035	NS	NS
S×*A*×*M*	NS	NS	NS	NS	NS	NS	NS	NS

Significance of effects of water stress, amendment and AM inoculation on the measured variables are also shown

SBR sugar beet residue, *AMF* arbuscular mycorrhizal fungi, *s.e.d.* standard error of difference between two means, *NS* not significant at $P>0.05$

Discussion

The improvement found in *T. articulata* shoot growth with the sugar beet amendment is in accordance with previous studies showing that the addition of organic residues enhances plant survival and growth under semiarid conditions [8]. Moreover, the ability of SBR to stimulate plant growth and nutrition in a soil contaminated artificially with Zn in the laboratory has been demonstrated [28]. The SBR is an organic amendment rich in polysaccharide compounds and available P through the rock phosphate applied during the fermentation process. Thus, plants grown in the amended soil had higher P contents in their tissues than non-amended plants.

The AM inoculation did not affect *T. articulata* shoot growth significantly. This is not surprising since *T. articulata* is highly capable of establishing a mycorrhizal symbiosis with the native AMF present in the experimental soil. Roldán et al. [33] found that both the inoculation of seedlings with a mixture of three exotic AMF and the addition of composted residue to soil stimulated the growth of *Juniperus oxycedrus* (a Mediterranean Cupressaceae like *T. articulata*), irrespective of the water regime. In the present assay, however, no effect of the sugar beet amendment or AM inoculation was found under water stress for *T. articulata*, only a reduction in shoot growth directly related with the water stress.

In *C. maritimum* as well as in *T. articulata*, water stress diminished shoot growth, with no effect of any treatment occurring under these conditions. However, unlike *T. articulata*, the SBR did not improve *C. maritimum* growth. Likewise, the native AM fungus, adapted to survive under contaminated environmental conditions, showed low effectiveness at promoting growth of *C. maritimum*, despite increasing mycorrhizal colonisation of the roots. This finding corroborates the fact that root colonisation levels are often not related to mycorrhizal function [38]. In contrast, in a previous study, it was shown that the inoculation with *G. mosseae* stimulated the growth of a leguminous shrub in the same contaminated soil [7]. Under such environmental conditions, it seems that the ability of *G. mosseae* to take up phosphorus and stimulate the growth of host plants depends upon the specific plant.

Table 4 Effect of the addition of sugar beet residue and inoculation with *G. mosseae* on relative abundance of fungal phospholipid fatty acids and AMF neutral lipid fatty acid biomarkers and ratio of fungi to bacteria in the rhizosphere soil of *T. articulata* and *C. maritimum* grown in a heavy metal-polluted soil under two different levels of watering

	Fungi PLFA (nmol g^{-1})		AMF NLFA (nmol g^{-1})		Fungi/bacteria	
	Ta	Cm	Ta	Cm	Ta	Cm
Well watered						
Control	0.2	0.5	4.3	0.4	0.1	0.2
SBR	1.2	0.8	11.5	1.3	0.2	0.1
AMF	1.2	0.5	5.4	0.3	0.8	0.2
SBR + AMF	1.3	0.8	13.8	1.4	0.2	0.1
Water stressed						
Control	0.5	0.2	6.7	0.3	0.3	0.2
SBR	2.0	1.0	5.9	0.3	0.3	0.1
AMF	0.5	0.3	5.3	0.2	0.5	0.2
SBR + AMF	1.0	0.9	14.3	0.8	0.2	0.1
s.e.d.	0.4	0.2	3.1	0.3	0.2	0.1
ANOVA, *P* values						
Water stress (*S*)	NS	NS	NS	NS	NS	NS
Amendment (*A*)	0.001	<0.001	0.008	0.018	NS	0.015
Mycorrhiza (*M*)	NS	NS	NS	NS	NS	NS
S×*A*	NS	NS	NS	NS	NS	NS
S×*M*	NS	NS	NS	NS	NS	NS
A×*M*	NS	NS	NS	NS	0.012	NS
S×*A*×*M*	NS	NS	NS	NS	NS	NS

Significance of effects of water stress, amendment and AM inoculation on the measured variables are also shown

SBR sugar beet residue, *AMF* arbuscular mycorrhizal fungi, *s.e.d.* standard error of difference between two means, *NS* not significant at $P>0.05$

For both plant species, the addition of the SBR increased rhizosphere microbial biomass, according to fungal, bacterial and AM fungal indicators. Thus, increases for the NLFA 16:1ω5 (a common AM indicator) were found when the sugar beet residue was added in the rhizosphere of both plants. The application of organic amendments like SBR can have a positive effect on the proliferation of the natural AMF in both crop and natural systems [1, 20, 22]. It has been shown that organic amendments with high C to N ratio may stimulate extraradical growth of AM fungi and bacterial biomass [40]. Since AM fungi are not able to feed directly from the organic matter added, the stimulatory effects of the amendment on the development of AM fungi may be related to an improvement in soil aggregate stability by providing a more suitable physical growing space and/or to an increase in microbial activity [26, 35]. We have previously demonstrated the effectiveness of SBR for improving structural stability of a soil from this neutral mine tailing [8]. Moreover, AMF are able to exploit inorganic nutrients released by the mineralisation of organic matter, through the activities of mineralising microorganisms [24]. However, there are many reports that show a strong, negative impact on the presence of AM fungal populations and mycorrhizal colonisation when a composted organic amendment is added to the soil [19, 36]. Interestingly, under water stress, the SBR had no effect on the NLFA 16:1ω5 amounts in the *T. articulata* rhizosphere when added alone, but improved them when added in combination with the AM inoculum. Since the soil used in this study was not sterilised, one suitable explanation for this behaviour could be that, in the absence of the AM inoculum, *T. articulata* plants established a mycorrhizal symbiosis with native AM fungi present in the soil, which may be less resistant to water stress than the *G. mosseae* strain used as inoculant.

The NLFA 16:1ω5 is an indicator of the amount of AM fungal mycelium, since it has been shown to be a sensitive and specific marker for AM fungal storage lipids and has been found to correlate better with AM colonisation in field soil than the PLFA 16:1ω5 [31]. However, while the SBR + AMF treatment substantially increased the amounts of NLFA 16:1ω5 under well-watered conditions, it had no positive effect on the degree of root mycorrhization. Our results suggest that the application of SBR improved the mycelial growth in soil but not the root colonisation.

A predominance of gram-negative over gram-positive bacteria is often found in heavy metal-contaminated soils.

Table 5 Effect of the addition of sugar beet residue and inoculation with *G. mosseae* on enzymatic activities in rhizosphere soil of *T. articulata* and *C. maritimum* grown in a heavy metal-polluted soil under two different levels of watering

	Phosphatase (μmol g^{-1} h^{-1})		β-Glucosidase (μmol g^{-1} h^{-1})		Dehydrogenase (μg g^{-1})	
	Ta	Cm	Ta	Cm	Ta	Cm
Well watered						
Control	1.3	1.7	0.4	0.5	4.5	5.3
SBR	1.3	1.8	0.5	0.7	10.3	11.1
AMF	1.4	1.8	0.4	0.5	4.0	7.5
SBR+AMF	1.5	2.5	0.5	0.7	7.9	11.2
Water stressed						
Control	1.2	1.6	0.4	0.4	4.3	8.8
SBR	1.6	1.7	0.6	0.7	8.2	13.0
AMF	1.5	1.5	0.4	0.5	4.2	6.1
SBR + AMF	1.5	1.4	0.5	0.8	9.8	14.4
s.e.d.	0.1	0.2	0.1	0.1	2.0	2.4
ANOVA, *P* values						
Water stress (*S*)	NS	0.002	NS	NS	NS	NS
Amendment (*A*)	0.004	NS	<0.001	<0.001	<0.001	<0.001
Mycorrhiza (*M*)	0.011	NS	NS	NS	NS	NS
S×*A*	NS	NS	NS	NS	NS	NS
S×*M*	NS	0.014	NS	NS	0.040	NS
A×*M*	NS	NS	NS	NS	NS	NS
S×*A*×*M*	0.020	NS	NS	NS	NS	NS

Significance of effects of water stress, amendment and AM inoculation on the measured variables are also shown

SBR sugar beet residue, *AMF* arbuscular mycorrhizal fungi, *s.e.d.* standard error of difference between two means, *NS* not significant at $P>0.05$

This is often explained by gram-negative bacteria being able to use a greater variety of C sources and adapt quicker to adverse conditions such as those of polluted soil [9, 11]. However, in this study, the two bacterial groups seemed to have a similar presence in the rhizospheric soil. The SBR increased the PLFA related to both bacterial groups, with a prevalence of gram-positive bacteria in both plant species. Some reports have demonstrated that the presence of gram-positive bacteria has a strong stimulatory impact on the formation and functioning of the AM symbiosis [2]. Thus, the increased presence of gram-positive bacteria recorded in the soil amended could have modified the levels of fungal colonisation in roots. In addition, due to their strong cell walls, gram-positive bacteria are in general more tolerant to water stress than gram-negative bacteria [16]. In *C. maritimum* plants, the effect of SBR amendment on bacterial gram-negative- and gram-positive-related PLFA was considerably higher under drought conditions than under watering conditions. However, the addition of SBR increased the gram-positive-related PLFA to a greater degree than the gram-negative-related PLFA under drought conditions.

The addition of an organic amendment like SBR modifies the fungal/bacterial ratio in the soil. In fact, an amendment, like SBR (C/N=32), improves fungal growth more than bacterial growth [34]. Thus, although increases in the fungal/bacterial biomass ratio are thought to reflect enhanced ecosystem efficiency and food web complexity in soil systems [37], the SBR addition hinders the interpretation of our results in this way. In our experiment, the SBR produced differential effects on the fungal/bacterial ratio depending on plant species. Thus, fungal/bacterial biomass ratio did not vary in *T. articulata* when SBR was added alone. Nevertheless, in *C. maritimum*, the SBR addition provoked a decrease in the fungal/bacterial biomass ratio. These findings could be explained by the differential effect of SBR-borne microorganisms on the indigenous microbial community of both rhizospheres. It is generally considered that different plant species will exert strong selective pressures on microbial rhizosphere populations, since the quantity and variety of compounds lost by rhizodeposition vary from species to species [25]. Thus, different microbial communities would respond in different ways to the toxic effects of heavy metals, which can inhibit the uptake by soil microorganisms of nutrients provided by the SBR [13]. It is worth noting that water stress did not affect the fungal/bacterial biomass ratio in the rhizosphere of both plant species, despite the strong decrease in the bacterial

biomarker in response to drying. These findings could indicate that fungal microorganisms associated with the rhizospheres are highly resistant to water stress and grow at relatively low water potentials, while the bacterial communities are particularly susceptible to soil water status and stress.

Other authors have found that soil hydrolase activities are enhanced by the addition of organic amendments, through the improvement of microbial growth and soil moisture. In our study, the addition of the SBR resulted in significant increases in β-glucosidase activity related to the C cycle, in the rhizosphere of both plant species. Phosphatases are enzymes with a relatively broad specificity, capable of hydrolysing various organic phosphate esters, and are involved in the P cycle. Increased acid phosphate activity in the rhizosphere of plants may be due to a fungal secretion or an induced secretion by the plant roots, but so far, it seems that AM fungus does not excrete phosphatases very much, as pointed out by Joner and Johansen [23]. The response of acid phosphate activity to the treatments assayed varied with plant species, possibly due to the differences in root exudates between both plants. Thus, while in *T. articulata* both SBR and mycorrhizal inoculation provoked an increase in phosphatase activity under drought conditions, in *C. maritimum* phosphatase activity of *G. mosseae*-inoculated plants was decreased as a consequence of water stress. Our results show that the release of acid phosphatase into the rhizosphere in response to SBR and AM fungus did not improve P acquisition of plants. Dehydrogenase is an oxidoreductase closely correlated with respiratory activity in soil [41] and is only present in viable cells. This enzyme has been considered as a sensitive indicator of soil quality [29] and a valid biomarker to indicate changes in total microbial activity due to changes in soil management [10]. Thus, the increases observed for this enzymatic activity in both plant species when the sugar beet amendment was added make sense.

Conclusions

The growth promotion effect of fermented SBR varied with plant species, being beneficial only in *T. articulata* plants. The amendment stimulated all major rhizosphere microbial groups of both plant species, such as AM fungi, non-mycorrhizal fungi and bacteria, resulting in an increase in microbial biomass size and activity. The mycorrhizal inoculation did not affect plant growth, regardless of the water regime. The effectiveness of the amendment, with regard to increasing several microbial populations and their activity as well as plant growth, was largely restricted under drought, particularly in the plant species with a low degree of mycorrhizal colonisation such as *C. maritimum*. Thus, *T. articulata* would be a better choice in future reforestation and bioremediation projects including organic amendments.

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