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Restoring the plant productivity of heavy metal-contaminated soil using phosphate sludge, marble waste, and beneficial microorganisms

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ABSTRACT

Assisted natural remediation (ANR) has been highlighted as a promising, less expensive, and environmentally friendly solution to remediate soil contaminated with heavy metals. We tested the effects of three amendments (10% compost, C; 5 or 15% phosphate sludge, PS5 and PS15; and 5 or 15% marble waste, MW5 and MW15) in combination with microorganism inoculation (rhizobacteria consortium alone, mycorrhizae alone, and the two in-combination) on alfalfa in contaminated soil. Plant concentrations of Zn, Cu, and Pb were measured, along with proline and malondialdehyde production. The microbiological and physicochemical properties of the mining soil were evaluated. Application of the amendments allowed germination and promoted growth. Inoculation with the rhizobacteria consortium and/or mycorrhizae stimulated plant growth. PS and MW stimulated the production of proline. Inoculation of alfalfa with the rhizobacteria-mycorrhizae mixture and the application of MW allowed the safe cultivation of the legume, as shown by the low concentrations of metals in plant shoots. Zn and Pb concentrations were below the limits recommended for animal grazing and accumulated essentially in roots. Soil analyses showed the positive effect of the amendments on the soil physicochemical properties. All treatments increased soil pH (around 7), total organic carbon, and assimilable phosphorus content. Notably, an important decrease in soluble heavy metals concentrations was observed. Overall, our findings revealed that the applied treatments reduced the risk of metal-polluted soils limiting plant growth. The ANR has great potential for success in the restoration of polymetallic

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and acidic mining soils using the interaction between alfalfa, microorganisms, and organo-mineral amendments.

Introduction

Pollution by heavy metals leads to severe and diffuse contamination of soils and significant environmental concerns. Moreover, hazardous concentrations of metals in the soil may affect biodiversity by affecting soil fertility and microbial activity (Wuana and Okieimen, 2011; Antonkiewicz et al., 2018; Skowrońska et al., 2020). In recent decades, this pollution has become a worldwide concern for which finding a solution is becoming more important to protect and conserve soils for future generations.

Various techniques have been developed to remediate mining soils, with assisted natural remediation (ANR) being one of the most promising strategies. ANR has been highlighted as a reliable and low-cost green technology and has been postulated as an alternative to conventional phytoremediation techniques (Xiong et al., 2015). The ANR approach aims to reduce waste disposal and to revalue wastes by recycling organic and inorganic matter (Lombi et al., 2002; Xiong et al., 2015). ANR enhances microbial activity, plant colonization and development, and thus reactivates the natural attenuation mechanisms in soil (adsorption, precipitation, and complexation) (Pérez-de-Mora et al., 2006; Midhat et al., 2018). The efficiency of this strategy can be promoted by the assistance by certain microorganisms, in particular, plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF). These microorganisms possess growth-promoting traits, including phosphate and potassium solubilization, nitrogen fixation, specific enzymatic activity secretion, and production of phytohormone and biofilm, thus permitting the improvement of plant growth, fitness, and biomass to remediate heavy metal-contaminated soils. In addition, these microorganisms may reduce the deleterious effects of metals on plants (Rajkumar et al., 2012; Ma et al., 2019).

The application of organic and inorganic matter is common in ANR strategies (Kumpiene et al., 2008; González et al., 2012). Many amendments have been shown to neutralize pH, increase soil fertility, and promote the biological and chemical stabilization of heavy metals. These amendments include organic manures (Pérez-Esteban et al., 2014), biochar (Wiszniewska et al., 2016), biosolids (Brown et al., 2005), lime (Zornoza et al., 2013), phosphate by-products (Hakkou et al., 2016), recycled sewage sludge (Skowrońska, et al., 2020), and zeolites (Shi et al., 2009). Other studies suggest the use of organo-mineral waste from industries to reclaim and improve soil quality (Antonkiewicz et al., 2018; Skowrońska et al., 2020). In particular, the use of waste enhances the benefit of remediation techniques by giving them new uses (Ghosh and Singh, 2005). The application of phosphate by-products and marble waste and their effects on soil physicochemical properties has been the focus of a few studies (Kabas et al., 2012; Tozsin et al., 2014; Midhat et al., 2018). These amendments can be suitable for bioremediation of heavy metal contaminated and acidic soils. Therefore, more studies focusing on the suitability of phosphate sludge and marble waste combined with inoculation with selected microorganisms for restoration and phytostabilization of polymetallic mining soil are needed.

The current study was conducted to determine the potential of alfalfa (*Medicago sativa* L.), selected rhizobacteria and/or mycorrhizae, combined with phosphate sludge or marble waste, to stabilize and restore the safe productivity of metal-contaminated mining soil. This was accomplished by assessing plant growth, stress markers (proline and malondialdehyde production), metal accumulation, and soil physicochemical properties.

1. Material and methods

1.1. Study site

The Kettara mine is located approximately 32 km Northwest of Marrakech (31° 52′00 "N and 8° 9′00″ W; 500 m above sea level). This mine was operated for pyrrhotite extraction until 1982 (Essaifi, 2011) and was used from 1964 to 1981 to extract various metals such as Cu, Zn, Fe, Cd, producing more than 5.2 million tons of pyrrhotite (Hakkou et al., 2008). Although the ore reserves were still high, the mine was closed in June 1982 owing to the difficulties in pyrrhotite production and its use. During the operation period, more than three million tons of waste were stockpiled on about 37 ha without treatment giving rise to considerable environmental concern (Toughzaoui et al., 2015), being Zn, Cu, and Pb are the major heavy metals present in the 'Kettara' mine soil (Midhat et al., 2018; El Alaoui et al., 2019).

1.2. Soil and amendments sampling

Kettara mine soil samples (KS) were randomly collected in December 2018 from a depth of about 15 to 30 cm. Agricultural soil (AS) samples were collected from the Marrakesh region. The compost used in our study was produced from green waste. The phosphate sludge (PS) was obtained during processing steps that involve crushing/screening, washing, and flotation of fluorapatite. The powdered marble waste (MW) was collected from a private marble processing unit (co. Sous-marbre, Marrakech, Morocco), and used in the experiment without prior treatment. All samples were air-dried at ambient room temperature and stored until use. The physicochemical and microbiological characteristics of soils and amendments were determined at 0 and 75 days of the experiment (n=4).

1.3. Alfalfa germination in amended soils

A seed germination test was carried out to investigate the effect of the different amendments on *M. sativa* seed germination. Glass Petri dishes were prepared by adding 50 g of the soil of each treatment as described below (greenhouse conditions and inoculation treatments). Sterile seeds were placed in each Petri dish (40 seeds per plate) and incubated at 25 °C. Data are means (± standard error) obtained from three to four independent experiments, with 3–5 replicates for each experiment. After 5 days, the number of germinated seeds in each Petri dish was counted. The water level was adjusted daily by adding distilled water to avoid changes in the physicochemical properties of the soil. The germination percentage (GR), germination speed (SG), the mean time germination (MTG, day), and the average daily germination (ADG) were calculated by the following formulae (Mateos-Naranjo et al., 2011; Rostamikia et al., 2016):

$$GR = \frac{n}{N} * 100\%$$

where n is the number of germinated seed after 5 days; N is the total number of seeds.

$SG = \sum_i (\frac{n_i}{d_i})$ Where n is the number of germinated seed at the day di.

$$MTG = \frac{\sum_i n_i * d_i}{\sum_i n_i}$$

where n is the number of germinated seeds at the day d_i.

$$ADG = \frac{GR}{D}$$

where D is the number of days at the final germination.

1.4. Rhizobacteria and arbuscular mycorrhizal fungi inocula

Alfalfa plants were inoculated with 10 mL of rhizobacteria consortium composed of four native metal-tolerant strains isolated previously from metal-polluted soils of the semi-arid region of Marrakesh: *Proteus* sp. DSP1, *Pseudomonas* sp. DSP17, and two *Ensifer meliloti* strains, RhOL6 and RhOL8 (Raklami et al., 2019b). A second inoculation with these rhizobacteria was made 15 d after seed sowing. In contrast, the native AMF consortium had been isolated earlier from the Tafilalet palm (500 km southeast of Marrakesh) and it contained a mixture of native species: (i) *Glomus* sp. (15 spores/g soil), (ii) *Sclerocystis* sp. (9 spores/g soil), and (iii) *Acaulospora* sp. (one spore/g soil) (Meddich et al., 2015). The AMF consortium was multiplied by trap culture in pots using *Zea mays* L. as the host plant under controlled greenhouse conditions for three months. The consortium was subjected to the most probable number test (Sieverding et al., 1991) to determine its potential infectivity. Alfalfa plants were inoculated with 5 g (fresh weight) of *Z. mays* mycorrhizal roots placed in proximity to the host root system as described by Raklami et al. (2019a).

1.5. Greenhouse conditions, inoculation, and harvesting

The Kettara mine soil (KS) was first mixed with unpolluted agricultural soil (AS) at a ratio of 1:1 (W/W); thereafter 200 g of the compost (10% W/W with respect to culture soil) was added (the lowest concentration that triggered alfalfa seed germination). Then, two amendments were tested; phosphate sludge (PS) or marble waste (MW). These amendments were used at 5% or 15% (W/W). The germinated seeds, previously disinfected with sodium hypochlorite solution diluted 1/5 (V/V) for 5 min were sown in 2.2-L plastic pots filled with culture soil. The experimental design was divided into 4 groups with 4 replicates. Each group contained the five treatments described above (compost alone, 5% or 15% phosphate sludge, and 5% or 15% marble waste). In this assay, the concentration of amendments used was determined by taking into consideration the improvement of soil physicochemical properties (pH and electrical conductivity), decrease in phytotoxicity (germination test), and the literature (Gonzales, 2012; Benidire, 2018; El Alaoui, 2018). The first group (NI) was not inoculated (control). The second group was inoculated with rhizobacteria alone (PR), the third inoculated with mycorrhizae alone (M), and the fourth was treated with a mixture of rhizobacteria and mycorrhizae (PRM). Pots were placed in individual trays in a controlled greenhouse at Cadi Ayyad University under natural daylight (250–1000 µmol/m^2/sec). The temperature was maintained at 25/21 °C day/night and relative humidity of 40%-60%. Plants were irrigated with tap water (250 mL) twice a week to ensure and maintain the same water level for all treatments. The experiment used four replicates, each with 20 seedlings.

Plants were harvested 75 days after sowing. Shoot and root elongation, leaf number, and shoot and root dry weights were measured to evaluate growth. Half of the plants were harvested at the end of the light period, frozen with liquid nitrogen, ground to a fine powder with a mortar pestle, and stored at -80 °C for the enzyme assays.

1.6. Determination of proline and malondialdehyde content

Free proline content was determined according to the method described by Bates et al. (1973). Briefly, 0.5 g of shoot plant material was homogenized in 10 mL of 3% aqueous sulfosalicylic acid, and the homogenate was filtered. The filtered solution (1 mL) was reacted with 1 mL ninhydrin reagent and 1 ml of glacial acetic acid in a test tube for 1 h at 100 °C, and the reaction was stopped by submerging the tubes in an ice bath. Proline was extracted with 2 mL of toluene and mixed vigorously. The toluene phase was removed and absorbance was read at 520 nm using toluene as a reference.

Malondialdehyde was determined according to the method described by Savicka and Škute (2010). In brief, lipid peroxides were extracted from leaves (0.1 g FW) with 0.5 mL of trichloroacetic acid. After centrifugation (15,000 × g for 20 min), the chromogen was formed by mixing 1 ml of supernatant with 2.5 mL thiobarbituric acid. The mixture was incubated at 95 °C for 30 min and the reaction stopped by placing the tubes in an ice bath. The chromogen formed was measured at 450, 532, and 600 nm.

1.7. Determination of metal concentrations in plants

Shoot and root dry matter (0.5 g) was mineralized at 450 °C for 4 hr. Dilute HNO_3 (5%) was then added to the samples before returning to 450 °C for 1 hr. Concentrated HNO_3 (5 mL) was added to the residue, which was then dried in a sand bath. Subsequently, 5 mL of H_2O_2 were added to the resulting residue, and the final volume was adjusted to 10 mL with 5% HCl (Tauzin and Juste, 1986). Metal concentrations were measured using a UNICAM 929 Flame Atomic Absorption Spectrophotometer. The standard solutions of Zn, Cu, and Pb were prepared from $Zn(NO_3)_2 \bullet 6H_2O$, $Cu(SO_4) \cdot H_2O$, and $Pb(NO_3)_2$, respectively.

1.8. Analyses of amended soils after the phytoremediation experiment

To determine the effects of amendment and/or inoculation treatments, soil samples were analyzed before and after the experiment (n=4). Physicochemical properties were determined according to the standard procedures of the Official Methods of Soil Analysis (Aubert, 1978). Texture was determined using the Robinson pipette method combined with sieving (Baize, 1988). The pH was measured in a suspension of soil diluted 1:5 (W/V) with distilled water. Electrical conductivity (EC) was measured using a conductivity meter. Total calcium carbonate ($CaCO_3$) was determined using a Bernard calcimeter. Total organic carbon (TOC) and matter (TOM) were calculated according to the method described by Aubert (1978). Total nitrogen (TN) was determined according to Rodier (1984), while ammonium nitrogen (NH_4^+) was measured by the Kjeldahl method following standard procedures

Table 1 – Main characteristics of Kettara mine (KS) and agricultural soil (AS), compost (CO), phosphate sludge (PS), and marble waste (MW) used for the experiment.

	Sand (%)	Silt (%)	Clay (%)	pH	EC (mS/ms)	$CaCO_3$ (%)	TOC (%)	TOM (%)	TN (mg/g)	NH_4^+ (%)	P_{ass} (mg/L)	Extractable (mg/kg)			Soluble (mg/kg)			Bact (*10^4)	Fun (*10^4)
												Zn	Cu	Pb	Zn	Cu	Pb		
KS	84	7	9	2.1^e	7.5^a	2.7^c	1.1^c	2.0^c	0.3^c	0.01^c	BDL	519.6^a	964.1^a	110.6^a	93.3^a	73.9^a	24.0^a	BDL	BDL
AS	64	27	9	8.6^b	0.5^c	6.0^c	1.6^b	2.7^b	0.5^b	0.01^b	10.9^c	26.7^c	16.8^b	9.2^c	0.3^b	3.3^b	4.7^b	99^b	0.43^b
CO	ND	ND	ND	8.1^c	5.5^b	ND	13.0^a	22.5^a	1.6^a	0.23^a	145.1^a	96.2^b	27.5^b	19.8^c	1.9^b	6.1^b	2.7^b	32×10^{5a}	420.00^a
PS	63	33	3	6.4^d	0.9^c	17.8^b	0.7^d	1.1^d	0.2^c	0.01^c	45.0^b	80.9^b	13.3^b	80.4^b	0.3^b	3.8^b	2.0^b	7^c	BDL
MW	44	48	8	9.4^a	0.5^c	86.7^a	1.0^{cd}	1.8^{cd}	0.1^d	0.00^c	12.2^c	8.9^c	4.4^b	13.1^c	0.1^b	3.9^b	3.7^b	0.35^d	0.30^c

Means within the same column followed by different letters are significantly different at $p < 0.05$.
EC: electrical conductivity; TOC: total organic carbon; TOM: total organic matter; TN: total nitrogen; Pass: assimilable phosphorus; Bact: total bacteria (CFU/g); Fun: total fungi; ND: not detected; BDL: below detection limit; KS: Kettara mine soil; AS: agricultural soil; CO: compost; PS: phosphate sludge, and MW: marble waste.

of the French Association of Normalizations (AFNOR, NF-T 90–015, 1975). Available phosphorus (P_{ass}) was measured according to Olsen and Sommers (1982). Zn, Cu, and Pb concentrations in soils were determined in triplicate according to the AFNOR standard X 31–151. Available metal fractions were determined using 0.01 M $CaCl_2$ (1:10 soil-extracting ratio) (Pueyo et al., 2004). Microbiological analyses were performed according to the serial dilution spreading method. Total bacteria (Bact) were counted after 48 hr on Trypticase soy agar (TSA) (at 28 °C), while fungi (Fun) were counted on potato dextrose agar (PDA) after one week (at 25 °C). Bact and Fun numbers are in colony-forming units (CFU/g).

1.9. *Statistical analyses*

The fit of the data to a normal distribution for all properties measured was checked using the Kolmogorov-Smirnov test. The data were subjected to ANOVA (SPSS Statistics V21.0 Inc., Chicago, USA) to assess differences among treatments. Mean separation was determined using the SNK (Student-Newman-Keuls) test with COSTAT software. Differences were considered significant if $P<0.05$. Growth, metal uptake, proline-malondialdehyde production, and their association with treatments were subjected to principal component analyses (PCA) using XLStat software. Percentage contributions of principal component (PC) variables and percentage contributions of the observations to the PC are shown in Appendix A Tables S1 and S2, respectively.

2. Results

2.1. *Initial characterization of soils and amendments*

Characterizations of the Kettara mine soil and amendments are shown in Table 1. The Kettara soil had a low organic matter and carbon content, low nutrient content, high acidity, and high heavy metal content. In contrast, agriculture soil, compost, and mineral amendments had an alkaline pH, high organic matter content, high nutrient availability, and low metal content. Soil cultivable bacteria and fungi were undetectable in the Kettara soil, whereas the agricultural soil had 10^6 bacterial CFU and 0.42 fungal CFU/g of soil. The compost had the highest bacterial and fungal populations.

Characteristics indicated that the amendments are valid for remediating the contaminated soil, particularly the phosphate sludge and marble waste owing to their high amount of calcium carbonate that neutralizes the strongly acidic pH and reduces the fraction of heavy metals bioavailable to plants.

All the Kettara soil samples were amended with 10% compost as a source of nutrients. The characteristics of the mixture were then determined (Table 2). The addition of compost and amendments (phosphate sludge at 5% or 15% or marble waste at 5% or 15%), neutralized the strongly acidic pH of contaminated soils and increased organic matter, total nitrogen, and plant nutrients. Initial measurements of the physical, chemical, and microbiological parameters after the application of the amendments showed that soil texture was very homogenous with a sandy loam texture for all treatments. After application of the amendments, soil texture was very homogenous with a sandy loam texture for all treatments. Soil pH was less acidic (pH was around 7), mainly owing to the buffering effects of compost, the organic matter from the agricultural soil, phosphate sludge, and the high content of $CaCO_3$ in the marble waste. EC did not always decrease and if it did the change was not significant. The addition of compost increased TOC in all treatments and the increased in TOM improved soil fertility and structure. Accordingly, the application of this amendment also improved the mineral content in the soil, total nitrogen, ammonium nitrogen, and assimilable phosphorus in the metal-contaminated soil.

Concentrations of Zn, Cu, and Pb were much higher in the Kettara soil than in agricultural soil (Table 1). Despite the high amount of trace elements in mining soil, the application of organo-mineral amendments phosphate sludge and marble waste substantially reduced concentrations of both soluble and extractable metals (Table 2). The soluble fractions of Zn, Cu, and Pb were much lower in soils amended with phosphate sludge and marble waste as compared to the compost treatment. The total content of Cu exceeded the advisory threshold for agricultural soils (100 mg/kg soil) (Adagundo et al., 2018). However, the concentration of soluble (available) Cu was low, perhaps due to the alkalinization caused by the addition of the amendments and/or binding to organic matter following compost addition (e.g. $CaCO_3 + 2H^+ \rightarrow Ca^{2+} + CO_2 + H_2O$). Similarly, the total concentration of Pb, although relatively high, was below the advisory threshold for agricultural soils and amendments (60 mg/kg soil) (Adagundo et al., 2018). In this respect, it is important to note that while dilution alone will decrease metal concentrations; the amendments also reduce their availability.

The applications of both organic and mineral amendments restored soil microflora with an increase in total bacteria and fungi (Table 2) compared to the KS intact contaminated mining soil (Table 1). All the plant substrates contained bacterial populations, with this being at the highest levels in substrates containing the phosphate sludge. Furthermore, the treatment with 15% marble waste and 5% phosphate sludge showed the

Table 2 – Main characteristics of different treatments before the experiment.

	Sand (%)	Silt (%)	Clay (%)	pH	EC (µS/ms)	$CaCO_3$	TOC (%)	TOM (%)	NT (mg/g)	NH_4^+ (mg/g)	P_{ass} (mg/L)	Extractable (mg/kg) Zn	Cu	Pb	Soluble (mg/kg) Zn	Cu	Pb	Bact (*10^4)	Fun (*10^4)
C	72	22	6	8.6^a	0.5^b	3.1^d	2.8^a	4.8^a	0.7^a	0.04^a	44.7^d	381.0^a	553.7^a	50.3^b	11.8^a	3.1^a	4.1^{ab}	79^c	39.8^c
PS5	62	33	5	7.1^c	3.2^a	4.9^c	2.8^a	4.9^a	0.4^c	0.03^b	63.8^{ab}	315.3^b	344.9^c	39.8^b	1.9b^c	1.0^b	5.4^a	158^b	6.3^d
PS15	63	28	9	7.3^c	3.3^a	5.3^c	2.8^a	4.9^a	0.4^{bc}	0.04^a	67.0^a	283.6^b	263.4^d	73.3^a	2.0^{bc}	0.7^b	3.3^b	246^a	55.5^a
MW5	76	17	7	7.8^b	3.1^a	7.1^b	2.8^a	4.8^a	0.6^{ab}	0.03^b	55.6^c	186.6^c	448.2^b	50.3^b	3.6^b	1.4^b	5.5^a	40^d	43.0^b
MW15	78	19	3	7.9^b	3.1^a	13.3^a	2.8^a	4.8^a	0.4^{bc}	0.02^c	63.8^b	148.9^c	396.6^{bc}	89.3^a	0.9^c	1.1^b	3.6^{ab}	40^d	4.1^e

Means within the same column followed by different letters are significantly different at $p < 0.05$.
C: (50% agriculture soil + 50% Kettara mining soil) + 10% compost; PS5: C+5% phosphate sludge; PS15: C+15% phosphate sludge; MW5: C+5% marble waste, and MW15: C+15% marble waste.

Table 3 – The effect of amendments applied on germination rate (GR), speed germination (SG), meant time germination (MTG) and average daily germination (ADG) of alfalfa seeds.

Treatment	GR (%)	SG	MTG (day)	ADG
C	66 (4)^c	13 (1)^c	4 (0)^a	13 (1)^c
PS5	80 (7)^b	22 (2)^b	4 (0)^{ab}	16 (1)^b
PS15	94 (1)^a	35 (2)^a	3 (0)^b	19 (0)^a
MW5	93 (5)^a	24 (2)^b	4 (0)^{ab}	19 (1)^a
MW15	95 (3)^a	34 (2)^a	4 (0)^b	19 (1)^a

Means (±standard deviation) within the same column followed by different letters are significantly different at $p < 0.05$.

lowest concentrations of fungi (around 10-fold lower than the rest of the substrates).

2.2. *Germination of alfalfa seeds in the amended soils*

Germination parameters were investigated to assess the effects of the amendments applied on alfalfa germination and seedling growth, and thereby to evaluate the potential to use metal-contaminated mining soils and wastes to produce alfalfa. The application of organo-mineral amendments significantly increased the germination parameters (GR, SG, and ADG) (Table 3). The maximum germination percentage (95%) was observed in seeds grown in Kettara soil containing 15% marble waste, whereas the lowest GR (65%) was recorded in soil amended with the compost alone. Moreover, the highest speed germination (SG) was recorded in the case of seeds germinated in the soil amended with phosphate sludge. As with GR, SG was lowest in the C treatment. The ADG reflects a measure of the rate and time-spread of germination. The lower the value, the quicker the germination. In the present study, the highest ADG was reported in seeds treated with compost (C). The lowest value was recorded in seeds grown in soil amended with 15% phosphate sludge (3.4 days) and 15% marble waste (3.6 days) in comparison to the non-treated soils (germination was delayed up to 4 days in average). These two treatments (PS15 and MW15) also had the highest ADG. The overall results presented in Table 3 indicate that the application of compost triggered alfalfa seed germination, while the application of phosphate sludge and marble waste improved it. This is important because the choice of plant suitable for remediation, germination, and establishment of the plant is a constant bottleneck in the success of phytoremediation projects.

2.3. *Evaluation of plant growth parameters*

The effects of organo-mineral amendments alone or combined with microorganisms (rhizobacteria or mycorrhizae) on alfalfa growth are illustrated in Fig. 1. The application of the organo-mineral amendments reduced the metal fraction available to plants, which allowed the growth and development of alfalfa in contaminated soil. In the control soil without amendments, inoculation with rhizobacterial or mycorrhizae consortia increased SL and SDW two-fold and inoculation with PRM three-fold. When amendments were added, even without inoculation, two- to three-fold increases in both shoot and root parameters were observed. Moreover, the combination of both treatments (material amendments and inoculation with rhizobacteria and mycorrhizae) produced the greatest increases in shoot parameters. The overall results reveal that the application of the dual inoculation rhizobacteria-mycorrhizae combined with 15% marble waste showed the highest shoot and root lengths, shoot fresh weight, and the total number of leaves (up to a five-fold increase in SDW as compared to the absolute control; plants without amendments and inoculation).

2.4. *Proline and malondialdehyde content in plants*

Accumulation of the stress biomarkers proline and MDA were measured in alfalfa shoots (Table 4). All inoculations promoted alfalfa growth and alleviated the stress caused by the toxic mining soil. Application of phosphate sludge and marble waste increased the content of proline in plant shoots. Inoculation with rhizobacteria or mycorrhizae stimulated the production and accumulation of this stress biomarker. Proline concentrations in alfalfa shoots ranged from 887 to 8144 µmol/g DM, with the highest concentration in plants grown in soil amended with MW15 and inoculated with mycorrhizae and in those grown in soil amended with PS5 and inoculated with the rhizobacteria-mycorrhizae complex. The lowest concentration was in plants grown in unamended soil, independent of the inoculation treatments. The application of marble waste, phosphate sludge, and rhizobacteria or mycorrhizae had no clear effect on MDA accumulation (Table 4).

2.5. *Metal uptake by plants*

The metals tended to accumulate in roots (Fig. 2). Zn and Pb concentrations in shoots were below recommendations for animal grazing (500 mg Zn and 100 mg Pb/kg) (Fig. 2). Cu concentration remained below the limitations for grazing livestock (40 mg Cu/kg) in PS15 inoculated with rhizobacteria, and C, PS5, MW5, MW15 inoculated with the rhizobacteria-mycorrhizae mixture. Treatments with phosphate sludge or marble waste and non–inoculated or inoculated with mycor-

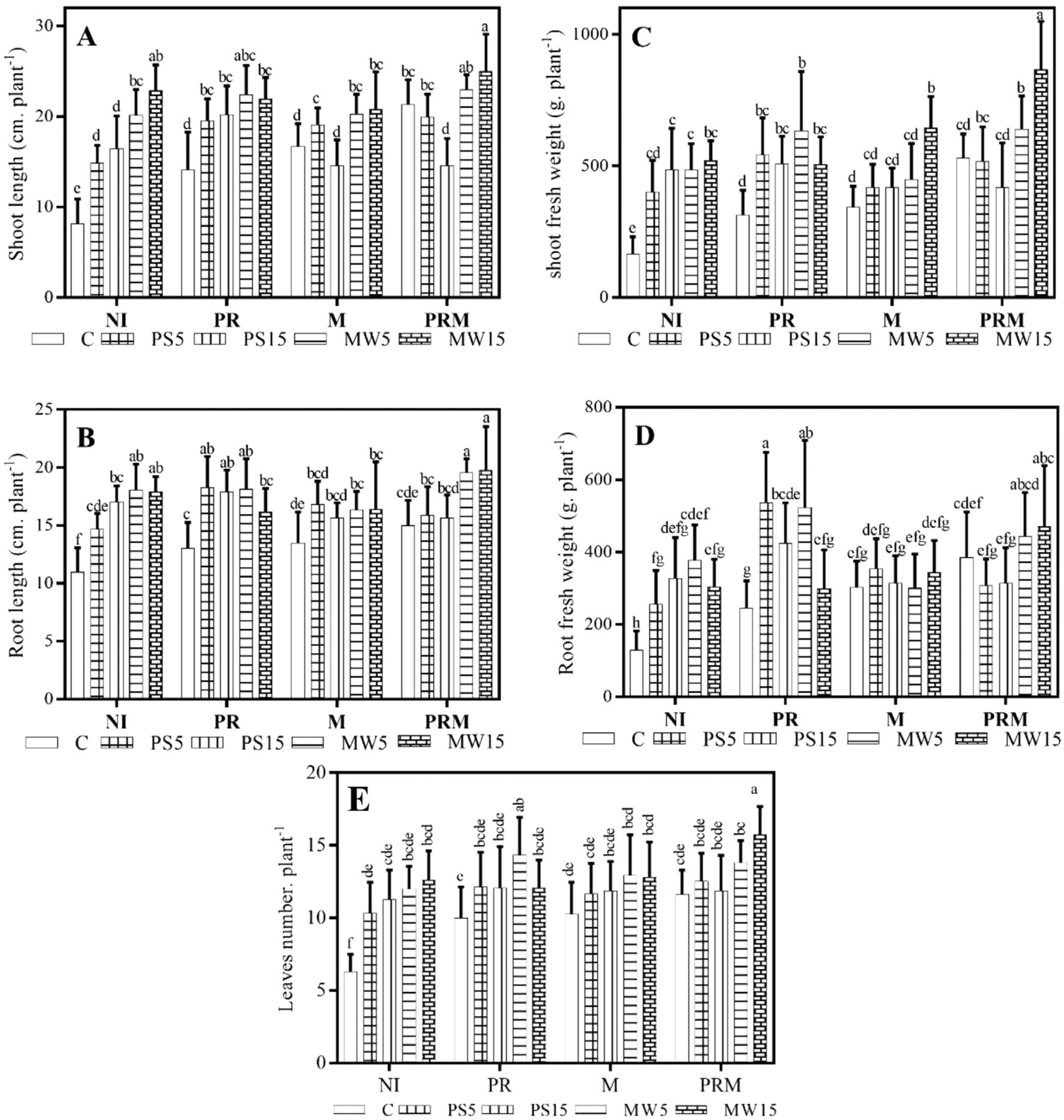

Fig. 1 – Shoot and root lengths (A and B), weights (C and D) and total leaves number (E) of alfalfa shoot subjected to different treatments. NI: non-inoculated; PR: Inoculated with the bacterial consortium; M: inoculated with mycorrhizae; PRM: inoculated with the mixture bacteria-mycorrhizae consortia. Means (± standard deviation) within the same graph followed by different letters are significantly different at $p < 0.05$.

rhizae displayed a high accumulation of trace elements, especially Cu.

2.6. *Principal component analyses*

The PCA analysis was carried out to evaluate the relationship between the studied treatments and several predictor variables, and to establish the variables with a prevalent influence on plant growth and metal accumulation. The PCA exhibits that treatments (in blue) and variables (in red) were associated with PC1 and PC2 (58%), of which PC1 was the major component (41%) (Fig. 3, **Appendix A Tables S1 and S2**). Treatments resulting in more growth, greater proline and MDA production, and lowest Cu accumulation are on the right side of the first axis (PC1). In contrast, the control treatment without inoculation (C-NI) showed lower growth, less proline and MDA production, and high accumulation of Cu in the shoot (left side of the first axis). In parallel, the treatments on the vertical axis corresponded to intermediate growth, proline and MDA production, and moderate metal accumulation. The overall results of this analysis showed that 5M+PRM and 15M+PRM were the best treatments in terms of plant growth, with proline and MDA production allowing safe cultivation of alfalfa grown in the mine soil since these displayed the lowest levels of metal accumulation.

2.7. *Physicochemical and microbiological analyses*

One of the main objectives of the assisted natural phytoremediation was to assess the influence of the diverse amendments and inoculation applied on contaminated mine soil pa-

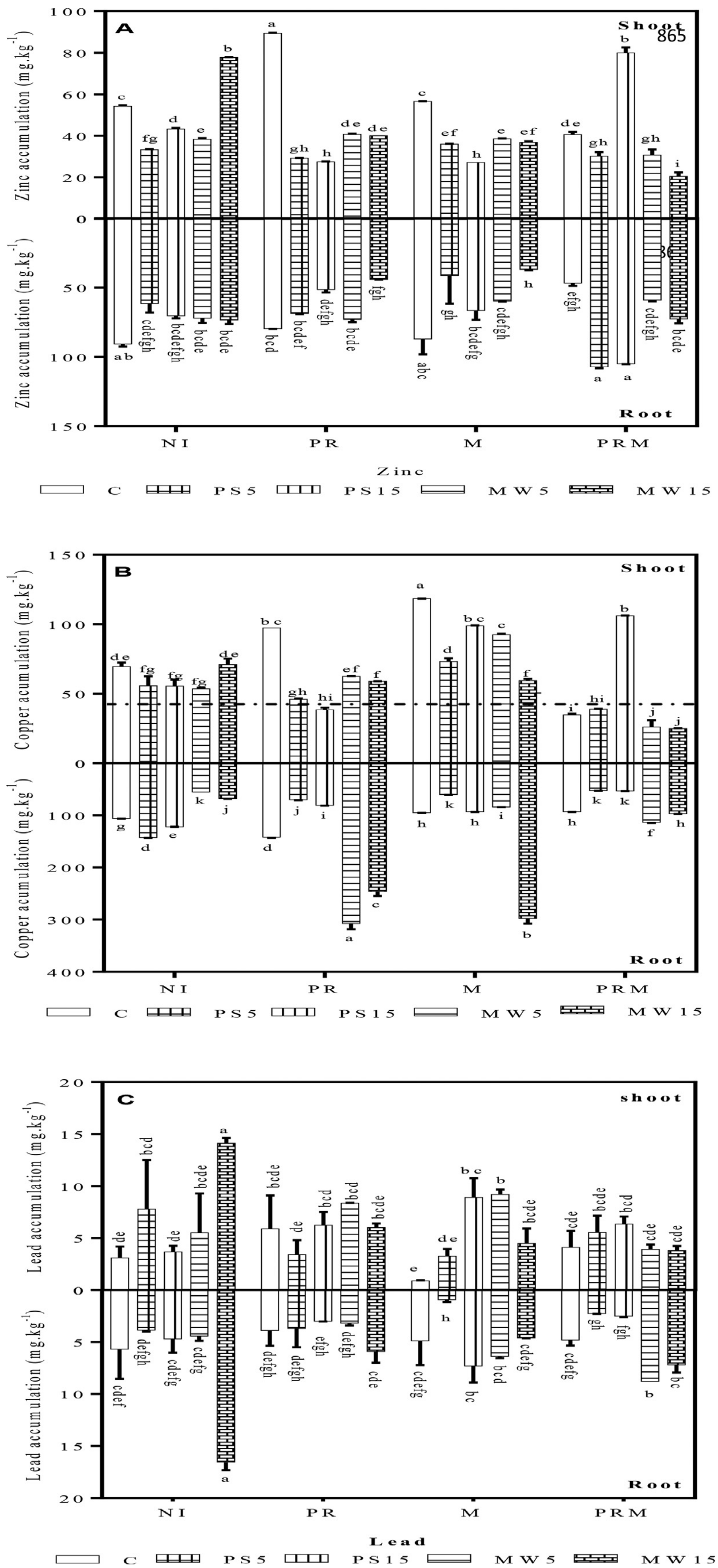

Fig. 2 – Zinc (A), cupper (B) and lead (C) accumulations in shoots and roots of *M. sativa* submitted to different treatments. Means (± standard deviation) within the same graphic followed by different letters are significantly different at $p < 0.05$. The dashed line in Fig. 2B represents the upper limit recommended for animals grazing.

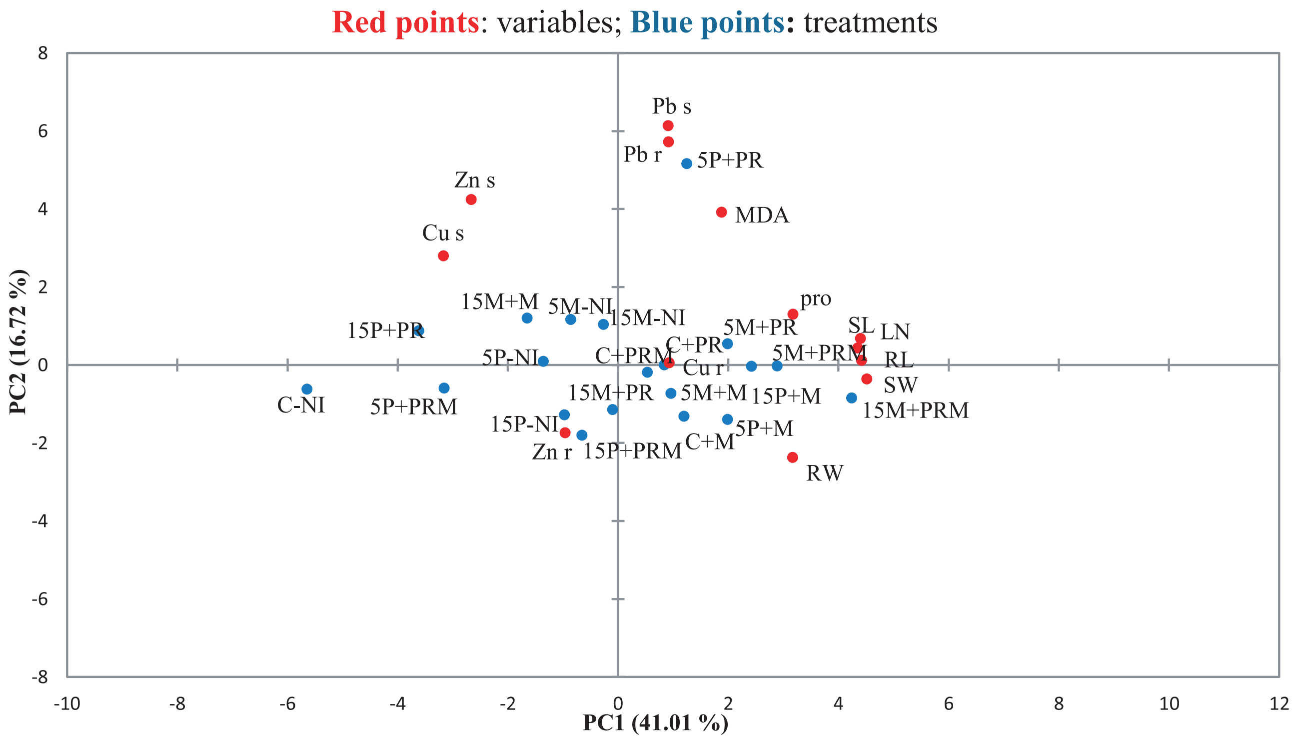

Fig. 3 – Principal component analysis (PCA) of alfalfa subjected to different treatments. The growth, proline-MDA production, and metals accumulation are represented in red. The treatments are given in blue. SL: shoot length; RL: root length; SW: shoot fresh weight; RW; root dry weight; NL: leaves number; pro: proline production; MDA: malondialdehyde production; Cu s, Zn s and Pb s: accumulation of copper, zinc and lead, respectively, in alfalfa shoot; Cu r, Zn r, and Pb r: accumulation of copper, zinc, and lead, respectively, in alfalfa root.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4 – Free proline and malondialdehyde (MDA) content in *M. sativa* shoots subjected to different treatments.

Treatment		Proline (μmol/ g DM)	MDA (μmol/ g FM)
NI	C	887 (20)[k]	0.01 (0.00)[b]
	PS5	2917 (48)[h]	0.01 (0.00)[b]
	PS15	1440 (33)[j]	0.01 (0.00)[b]
	MW5	4991 (132)[de]	0.02 (0.00)[ab]
	MW15	5288 (12)[cd]	0.02 (0.00)[a]
PR	C	2099 (31)[i]	0.01 (0.00)[ab]
	PS5	3653 (129)[g]	0.02 (0.00)[ab]
	PS15	4831 (209)[de]	0.02 (0.00)[ab]
	MW5	5728 (1043)[bc]	0.01 (0.01)[ab]
	MW15	6177 (35)[b]	0.01 (0.00)[b]
M	C	1349 (101)[j]	0.02 (0.00)[ab]
	PS5	4964 (1)[de]	0.01 (0.00)[ab]
	PS15	4287 (37)[f]	0.02 (0.00)[ab]
	MW5	2913 (272)[h]	0.01 (0.00)[ab]
	MW15	7797 (86)[a]	0.02 (0.00)[a]
PRM	C	1699 (142)[ij]	0.01 (0.00)[ab]
	PS5	8144 (42)[a]	0.01 (0.00)[ab]
	PS15	5410 (5)[cd]	0.02 (0.00)[ab]
	MW5	4642 (43)[ef]	0.02 (0.00)[ab]
	MW15	5220 (32)[cde]	0.02 (0.00)[ab]

Means (±standard deviation) within the same column followed by different letters are significantly different at $p < 0.05$. DM: dry matter; FM: fresh matter.

rameters to determine the phytoremediation capacity of the proposed system.

After 75 days, the amended soils retained the same features at the beginning of the experiment. The neutral pH, lower conductivity, TOC, and TOM increases were generally observed in all the inoculated and non-inoculated treatments (Table 5). It is noteworthy to indicate a tiny reduction in terms of total and ammonia nitrogen. Assimilable phosphorus fluctuated depending on the inoculation and the amendment applied. The pH, which varied between 4 and 7, coincided with the effective precipitation (and adsorption) range for most of the metals. This was evident in our study with the lowest amount of available trace elements in soil solution at the end of the experiment. Inoculation generally did not affect concentrations of elements in the soil. The applied amendments maintained a high pH and low metal availability even after 75 days, allowing, therefore, subsequent plant establishment and growth. Importantly, we observed a 10-fold decrease in total fungi in soil compared to the beginning of our experiment because of soil toxicity. However, the total bacteria in soils were improved especially in the treatments inoculated with rhizobacteria.

3. Discussion

Trace element bioavailability and solubility are more critical in bioremediation techniques than the total concentration of these elements in the contaminated soil because they represent the most labile fraction, subject to leaching

Table 5 – Main characteristics of different treatments after 75 days of the experiment.

Treatments		pH	EC (μS/ms)	TOC (%)	TOM (%)	TN (mg/g)	NH_4^+ (%)	P_{ass} (ppm)	Soluble (mg/kg)			Bact (*10^6)	Fun (*10^3)
									Zn	Cu	Pb		
NI	C	4.1^{f}	3.5^{bcde}	3.4^{de}	5.9^{de}	0.4^{bc}	0.03^{a}	84.3^{b}	0.2^{fg}	2.8^{c}	2.0^{i}	69^{ghi}	67^{d}
	PS5	6.2^{bcd}	3.2^{cde}	4.5^{c}	7.8^{c}	0.2^{ef}	0.02^{fg}	69.5^{cd}	2.0^{d}	2.8^{c}	5.4^{d}	66^{hi}	74^{cd}
	PS15	6.7^{abc}	3.1^{de}	3.8^{d}	6.5^{d}	0.2^{fg}	0.02^{fg}	83.8^{b}	2.0^{fg}	3.0^{bc}	3.5^{gh}	55^{i}	80^{c}
	MW5	7.4^{a}	3.6^{bcde}	5.0^{c}	8.6^{c}	0.2^{ef}	0.03^{abc}	39.8^{g}	2.0^{d}	1.7^{de}	4.2^{fgh}	$84f^{g}$	63^{d}
	MW15	7.5^{a}	3.2^{cde}	3.9^{d}	6.7^{d}	0.2^{fg}	0.02^{g}	31.5^{g}	6.7^{b}	3.4^{b}	3.7^{efgh}	69^{ghi}	36^{f}
PR	C	5.2^{e}	4.1^{bc}	4.7^{c}	4.1^{c}	0.2^{fg}	0.02^{de}	55.6^{f}	0.4^{fg}	3.0^{c}	4.1^{efgh}	64^{hi}	73^{cd}
	PS5	6.5^{abcd}	3.3^{bcde}	3.8^{d}	6.5^{d}	0.4^{b}	0.03^{cde}	98.2^{a}	0.0^{g}	2.7^{c}	4.0^{efgh}	76^{gh}	80^{c}
	PS15	6.2^{bcd}	4.9^{a}	4.8^{c}	8.3^{c}	0.5^{b}	0.03^{abc}	104.8^{a}	0.3^{fg}	0.8^{fg}	4.5^{ef}	170^{a}	85^{c}
	MW5	7.4^{a}	3.7^{bcde}	2.4^{g}	4.1^{g}	0.2^{fg}	0.03^{a}	97.2^{a}	3.9^{c}	8.9^{a}	4.4^{efg}	107^{cde}	43^{ef}
	MW15	7.5^{a}	3.0^{de}	3.0^{ef}	5.2^{ef}	0.1^{g}	0.03^{cde}	41.1^{g}	0.0^{g}	0.7^{fg}	4.2^{efgh}	94^{def}	72^{cd}
M	C	5.9^{cde}	5.8^{a}	3.6^{de}	6.2^{de}	0.3^{de}	0.03^{abc}	55.7^{ef}	0.0^{g}	0.2^{fg}	4.2^{efgh}	70^{ghi}	32^{f}
	PS5	6.6^{abcd}	5.1^{a}	1.8^{h}	3.1^{h}	0.2^{fg}	0.02^{de}	63.8^{de}	0.0^{g}	0.3^{g}	8.6^{a}	76^{gh}	82^{c}
	PS15	6.6^{abcd}	4.2^{b}	2.7^{fg}	4.6^{fg}	0.2^{ef}	0.03^{bcd}	56.5^{ef}	0.1^{g}	0.9^{f}	6.5^{c}	122^{b}	97^{b}
	MW5	7.4^{a}	3.1^{cde}	4.8^{c}	8.3^{c}	0.4^{bc}	0.03^{cde}	73.9^{c}	3.4^{c}	0.4^{g}	4.8^{e}	41^{j}	79^{c}
	MW15	7.5^{a}	2.9^{e}	6.3^{a}	10.9^{a}	0.3^{de}	0.02^{ef}	63.4^{de}	1.1^{ef}	0.1^{g}	3.4^{h}	42^{j}	49^{e}
PRM	C	5.7^{de}	3.0^{de}	2.5^{fg}	4.4^{fg}	0.5^{a}	0.03^{ab}	37.0^{g}	1.6^{g}	1.3^{e}	3.6^{fgh}	92^{ef}	262^{a}
	PS5	6.9^{ab}	4.0^{bcd}	2.7^{fg}	4.6^{fg}	0.3^{cd}	0.02^{cde}	39.2^{g}	3.3^{c}	1.9^{d}	5.9^{d}	108^{cd}	95^{b}
	PS15	7.3^{a}	3.5^{bcde}	1.1^{i}	2.0^{i}	0.2^{ef}	0.03^{fg}	52.0^{f}	0.6^{fg}	0.6^{fg}	7.4^{b}	96^{def}	97^{b}
	MW5	7.4^{a}	3.4^{bcde}	3.3^{de}	5.7^{de}	0.2^{fg}	0.03^{bcd}	83.0^{b}	0.1^{g}	0.4^{fg}	4.2^{efgh}	112^{bc}	34^{f}
	MW15	7.4^{a}	3.0^{de}	5.6^{b}	9.6^{b}	0.1^{g}	0.02^{fg}	62.0^{def}	10.3^{a}	0.7^{fg}	5.5^{d}	98^{def}	41^{ef}

Means within the same column followed by different letters are significantly different at $p < 0.05$.

and absorption by plants and microorganisms (Adriano, 2001; Skowrońska et al., 2020).

Soils contaminated with metals in general or Kettara mine soil, in particular, are unreceptive environments for plant growth owing to low nutrient availability, low carbon and organic matter content, low microbial diversity, and high acidity (pH ≈ 2) and heavy metal content. These soils pose environmental hazards, such as toxic metals reaching water sources through the wind, water erosion, and leaching. Assisted natural remediation, with organo-mineral amendments or microorganisms, offers a sustainable alternative for the restoration of contaminated soils, and is socially accepted and economically and environmentally friendly. However, few studies have focused on phytoremediation assisted by amendment addition and/or microorganisms (rhizobacteria and/or mycorrhizae) to improve soil quality and rehabilitate sites contaminated by metals (Zornoza et al., 2013; Navarro-Torre et al., 2017; Paredes-Páliz et al., 2018; González et al., 2019; Skowrońska et al., 2020).

The use of amendments (compost, phosphate sludge or marble waste) alone or in combination with native indigenous rhizobacteria and/or mycorrhizal fungi consortia to limit the transport of trace metals from roots to shoots of alfalfa was evaluated here. Our study demonstrated that the addition of mineral amendments (phosphate sludge and marble waste) allowed a better percentage of germination permitting an increase in germination rate, as well as the enhancement of alfalfa growth. This can be attributed to improved fertility and structure of the contaminated mine soil, in addition to the reduction of phytotoxicity related to metals. Midhat et al. (2018) showed that marble waste applied at the rate of 25%, 50%, and 75% (W/W) on contaminated mine soil allowed better germination, installation, and development of plants. Additionally, single or dual inoculation with indigenous rhizobacteria or mycorrhizae promoted growth, in terms of shoot and root lengths, weights, and number of leaves. This improvement in the capacity of the inocula to display different traits could be attributed to nitrogen fixation and phosphate solubilization by these microorganisms, and phytohormone production (Raklami et al., 2019b). The application of indigenous microorganisms in bioremediation constitutes a rewarding strategy compared to non-native or exotic measures. Chang et al. (2014) demonstrated that the use of native bacterial strains for phytoremediation of saline soil promotes (more than exotic microorganisms) the growth and development of plants. Furthermore, the positive effect of rhizobacteria and mycorrhizae on plant growth cultivated under metal stress has been documented (El Faiz et al., 2015; Navarro-Torre et al., 2017; Raklami et al., 2019b). There are various mechanisms by which plants reduce metal toxicity, including (i) the production of metal-binding factors such as cysteine-rich proteins, Cys-X-X-Cys-motif, and peptides that avidly bind, immobilize, sequester, and detoxify the heavy metals such as copper and cadmium to reduce their concentration to a physiological or nontoxic level, (ii) exclusion of toxic heavy metals from cells by ion-selective metal transporters, (iii) and excretion or compartmentalization (Hu et al., 2001; Emamverdian et al., 2015). Two mechanisms through which plants detoxify heavy metals are the production of proline and malondialdehyde (Sánchez-Pardo and Zornoza, 2014; Kanwal et al., 2015; Wang et al., 2018).

The application of phosphate sludge and marble waste stimulated the production of proline in the plant and inoculation further enhanced its production. Several studies have reported that proline and MDA production were stimulated by the presence of metals in both shoots and roots in plants without symbionts to a greater extent than with symbiotic microorganisms (Sánchez-Pardo and Zornoza, 2014). Kanwal et al. (2015) found that zinc and cadmium toxicity led to an increase in proline content in the shoot of non-mycorrhizal alfalfa plants, whereas mycorrhization tended to decrease it. Sánchez-Pardo and Zornoza (2014) reported that cultivation of *Lupinus albus* under Cu stress induced the production of MDA as a marker of membrane damage due to lipid peroxidation, while inoculation with *Bradyrhizobium* sp. decreased it. The inoculation of soybean (*Glycine* max L.) did not affect MDA production. Wang et al. (2018) observed that the application of pine biochar, kaolin, and triple superphosphate increased the content of MDA in *Buxus microphylla* and *Salix* sp. grown in soil contaminated with Ni, Zn, Cu, and Cd. The MDA concentration provides insight into lipid peroxidation and, therefore, oxidative stress. The lack of significant differences in MDA in the present study suggests that the application of the organo-mineral amendments or native microorganisms reduces oxidative stress. The mitigation of oxidative stress can be attributed to the secretion of low molecular weight metal-sequestering compounds, such as ascorbic acid, thiols, and proline (Hossain et al., 2012).

Important aspects of this study include a potentially safe way to use metal- contaminated-mining soils assisted by phosphate sludge, marble waste amendments, and beneficial microorganisms to produce alfalfa. The phytostabilization potential of alfalfa has been widely reported since this plant is considered a non-hyperaccumulator and metals are concentrated mainly in root tissues (Pajuelo et al., 2011; Mingorance et al., 2017; Midhat et al., 2018). The accumulation of metals in alfalfa plant tissues is root > stem > leaf (Pajuelo et al. 2007; Midhat et al., 2018). Application of compost, phosphate sludge, and marble waste decreased metal translocation to shoots; Zn and Pb accumulation values fell far below the threshold for animal grazing (500 mg Zn and 100 mg Pb/kg). Midhat et al. (2018) similarly reported that the application of marble waste at the rate of 25% and 50% decreased the translocation of metals to alfalfa stems and shoots. The inoculation of alfalfa (grown in amended soil) with rhizobacteria alone or in combination with mycorrhizae allowed safe cultivation for 75 days, with the metal content below the threshold recommended for animals grazing. Indeed, Cu concentrations were below the limits (40 mg/kg) in PS15 inoculated with rhizobacteria, and in C, PS5, MW5, MW15 inoculated with the rhizobacteria-mycorrhizae mixture compared to control plants. These findings can be attributed to the buffering effects of compost, agricultural soil, phosphate sludge, and the high content of $CaCO_3$ in marble waste, that allow that increase soil pH, which reduces metal bioavailability. It is important to note that the dilution resulting from addition of the amendments alone will also have a positive effect on reducing the content of metals in soil that directly influences the amounts of metals accumulated by alfalfa.

Precipitation and adsorption of metals to soil particles and organic matter will reduce their uptake by plant roots and subsequent translocation to the aerial parts (Mignardi et al., 2013; Lwin et al., 2018). In addition, the applied rhizobacteria can produce exopolysaccharides (which can bind metals and make them less available) forming a dense biofilm around the root system; this biofilm may act as a barrier to the absorption of metals by plant roots (Raklami et al., 2019b). Mycorrhizae can decrease metal uptake from soils by solubilizing metals *via* changes in soil pH, which may be a defense strategy adopted to avoid or escape the negative impact of high soil metal concentrations (Rajkumar et al., 2012; Ma et al., 2016). Chelation/immobilization of metals by extraradical mycelium, glomalin, or exudates can sequester metals (Wang et al., 2007). Plants at a high population density may also better tolerate contaminants because they are essentially in competition, such that tolerance may be less at the lower seeding rate likely in the field.

The soil physicochemical analyses before the experiment showed that the application of the organo-mineral amendment positively affected the contaminated mine soil. The addition of agricultural soil and organic and/or mineral amendments had a significant positive effect on soil pH, total carbon and organic matter, nitrogen, phosphorus, and more importantly a significant decrease in soluble and extractable Zn, Cu and Pb concentrations. The application of such organo-mineral amendments has been previously reported for both types of mine soil (acidic and alkaline) since they improve soil fertility and reduce heavy metals availability and toxicity (Kabas et al., 2012; Zornoza et al., 2012; Midhat et al., 2018). The application of agricultural soil and compost in the restoration of mining soil has several advantages including the increase in soil pH, carbon and organic matter, soil fertility, cation exchange capacity, plant nutrient availability, soil biodiversity and biological activity, and soil water maintenance (Pardo et al., 2011; Sidhu et al., 2016; Navarro-Pedreño et al., 2017). Adsorption, sequestration, precipitation, and complexation are the main mechanisms during the phytostabilization process (Mignardi et al., 2013; Lwin et al., 2018). Bacterial extracellular complexation and intracellular accumulation can reduce the uptake of metals by plants (Vivas et al., 2006). Bai et al. (2014) showed that the complexation of Pb^{2+} with carboxyl, hydroxyl, carbonyl, amido, and phosphate groups on the cell wall and in extracellular polymeric secretions and the formation of precipitates of $Pb_5(PO_4)_3OH$, $Pb_5(PO_4)_3Cl$ decreased the bioavailability of Pb in soil. Results of the present study show that the applied treatments reduced toxicity that precludes or impedes the introduction of plants in contaminated mine soil.

The physicochemical analyses after the end of the experiment revealed an important decrease in soil pH in non-amended control (C) treatment, while it remained neutral in soils amended with phosphate sludge and marble waste. The increase in organic carbon in the rhizosphere would be influenced by exudates through the metabolism and physiological activities of plant roots induced by microorganism inoculation (Wu et al., 2006). These microbes can produce a variety of growth-promoting substances, including indole acetic acid, cytokinins, gibberellins, and B vitamins, which stimulate root exudate production (Asari et al., 2016; Massalha et al., 2017; Backer et al., 2018; Benidire et al., 2020). The soluble metals were, in general, low in most cases throughout the experiment. Biological analyses showed an enhancement of total bacteria and surprisingly a significant decrease in the total fungi community. This decrease could be partly related to the increases in total bacteria, soil pH, and nitrogen content (Högberg et al., 2007; Rousk et al., 2009). A negative effect on microbial communities has been reported previously under higher metal concentrations in soils (Liao and Xie, 2007). Indeed, metal contamination also exerts selective pressure on soil microbial communities, leading to a shift in community structure and a decrease in functional diversity (Liao and Xie, 2007). For instance, high Cu concentration in soils has been negatively correlated with the total bacteria and fungi.

Use of wastes and/or residues of different industries such as phosphate sludge and marble waste, besides improving the phytoremediation process, help to compensate the cost of the implementation, adding extra value to these environmentally friendly remediation methods (Ghosh and Singh, 2005).

To visualize the differences among treatments with reference to growth parameters, the content of proline, MDA, bioaccumulation heavy metal content, and root colonization parameters, a PCA analysis was performed. The first PCA axis, which explained 41% of the variation, was highly correlated (right side) with plant growth parameters (shoot and root lengths, leaf number, and plant weight). These variables showed their stronger association with the 5 and 15% marble waste treatments combined with the inoculation with the rhizobacteria-mycorrhizae mixture (5M+PRM and 15M+PRM) 5M+PRM and 15M+PRM treatments. In contrast, the non-inoculated control treatment (C-NI) was separated into a distinct group (left side) and showed a negative correlation with the measured variables. The third group corresponding to other treatments occurred in intermediate positions. These findings showed that marble waste combined with the application of rhizobacteria-mycorrhizae mixture are factors driving to the safe cultivation of *M. sativa*. Studies are needed on the effect of these amendments in the field since plants could be more sensitive when they are at a lower density.

4. Conclusions

The application of phosphate sludge and marble waste had a positive impact on alfalfa growth in heavy metal-contaminated mining site soil. Single or dual inoculation of plants with rhizobacteria and mycorrhizae consortia resulted in further improvement. Proline production was identified as a target to improve plant tolerance of the heavy metals toxicity after the addition of mineral amendments. Application of marble waste at 15% (W/W) combined with the dual inoculation allowed safe cultivation of alfalfa in a 75-days experiment. Additionally, our findings demonstrate that the application of compost, phosphate sludge, and marble waste could be considered as a feasible technology and a potentially cost-effective approach during phytoremediation by ameliorating the soil health and fertility that could allow the subsequent introduction of other plant species. Future studies should include the critical impacts of soil conditions and the molecular mechanisms involved to further develop effective and efficient remediation strategies using soil amendments and plants.

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Appendix A. Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.06.032.

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