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The Role of Mycorrhizal-Assisted Phytomining in the Recovery of Raw Materials from Mine Wastes

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Abstract: In recent years, critical and secondary raw materials (CRMs and SRMs, respectively) have received great interest within the circular economy model. In this work, the mycorrhizal-assisted phytomining (MAP) system, composed of *Helianthus annuus*–arbuscular mycorrhizal fungus *Rhizophagus intraradices*–Zn-volcanic ashes, was applied in bioreactors for the recovery of CRMs (Sr, P) and SRMs (Cr, Zn, Cu, Mn, Rb, Ni) from mining wastes of the *Los Cóndores* mine (Argentina). Our results showed high bioaccumulation of Sr, P, Mn, and Zn in the aerial tissues, and a high root-to-shoot translocation for Mn (4.02) > Sr > P > Rb > Zn (0.84). Mycorrhization treatment increased the root-to-leaf translocation for Cr and P and prevented translocation towards flower tissues in most elements. The estimated bioextracting potential of the MAP system (290 plants) in a vegetable depuration module (VDM) ranged from 158 mg/m^3 P > Zn > Mn > 15.1 mg/m^3 Sr. We demonstrated a promising and cost-effective biotechnology applicable in agronomical practices, given the exclusion of toxic elements in flower parts, as well as for the recovery of CRMs and SRMs by hydrometallurgy from plant biomass.

Keywords: mycorrhizal-assisted phytomining; bioreactors; hydrometallurgy; resource recovery

1. Introduction

The circular economy represents a completely new concept for the life cycle of a product, which involves sustainable activities such as recycling and reuse. Nowadays, this model is replacing the traditional linear economy of "take—make—dispose" model given that the circular economy brings benefits not only to the environment through waste reduction but also to the economy through savings in raw materials [1,2]. Nowadays, the exploitation of new raw materials for the development of high-value-added products is not profitable, but these could be gained if efficient waste recycling technologies are applied. Specifically, the major anthropogenic sources of critical and secondary raw materials (CRMs and SRMs, respectively) in the environment include wastes from metalliferous mining or smelting industries [2]. Many chemical elements were declared of special interest in the fourth EU list [3] due to the growing demand in industry and the limited availability in nature, highlighting the abandoned mining sites as a source of SRMs and CRMs [2].

Phytomining biotechnology involves plant species capable of extracting chemical elements from subeconomic deposits or mineral wastes and accumulating them in plant biomass to subsequently recover the element in value [4]. Currently, phytomining is applied for the recovery of CRMs and SRMs from solid metal wastes, resulting in an excellent option

for a circular economy [5,6]. The application of phytomining benefits the environment by reduction of toxic effects [7], and it could be improved by incorporating beneficial microorganisms associated with the rhizosphere of target plants. In particular, the phytoextraction and phytoaccumulation capacities could be enhanced by the mutualistic association of these plants with root symbionts, such as the arbuscular mycorrhizal (AM) fungi [8]. These fungi generate a direct relationship between soil and roots, and therefore, they contribute to the bioavailability of chemical elements in soil [9]. Various reports have mentioned the abilities of AM fungi to uptake different elements from the soil, sequester them in their structures, or translocate them to their host plant. The mechanisms of bioaccumulation in the mycorrhizal association are highly dependent on the fungal and plant partner involved, as well as some environmental factors [10]. Previously, we obtained an efficient extraction of CRMs and SRMs from mining soil by using the mycorrhizal-assisted phytomining (MAP) system, comprised of "*Helianthus annuus–Rhizophagus intraradices*–Zn 350-volcanic ash" (Patent 130100620) [11] under laboratory conditions at Technological Readiness Level (TRL) 2 [5]. Also in this work, the bioextracting potential (BP) values were estimated after calibration of the MAP system in an engineering module depuration (vegetable depuration module, VDM) at TRL 6 [8], obtaining values of 2.417 g (K) > BP > 0.14 g (As) per m^3 of contaminated soil. Finally, the recovery of SRMs and CRMs by hydrometallurgical techniques was suggested as a viable and cost-effective option. However, the scaling-up of a pilot test or prototype technology at TRL 4 before TRL 5-6, to reach the maturity levels towards a real environment, is urgently needed [12].

In the present work, we used an innovative automatized technological prototype, called a bioreactor, which allowed us to validate the MAP system at TRL 4 [13] for the decontamination of polluted soil, and simultaneously, recovering useful CRMs and SRMs. The bioreactor permits the calibration of hydraulic variables of entry and exit of effluents and other physicochemical parameters. After the MAP system performance in the bioreactor, it can be followed with the harvest of chemical elements from plant biomass and purification with bio-hydrometallurgical technology for the recovery of SRMs and CRMs with high extraction efficiency and high purity degree. The microbial-assisted phytomining linked to hydrometallurgy techniques is an innovative and economical technology to mine waste treatment [1,7].

The aim of this work was to calibrate and validate the MAP system applied in the bioreactor at TRL 4 using mining wastes from the Los Cóndores mine (San Luis province, Argentina) for the recovery of SRMs and CRMs, and subsequent decontamination of polluted soil. Finally, a prediction of the performance of the MAP system in the VDM at TRL 6 containing mine residues was estimated.

2. Materials and Methods

2.1. Sampling Sites

The sampling campaign in the Los Cóndores mine (Concarán, San Luis province, Argentina) was carried out within the framework of the Horizon 2020 ERA MIN BioCriticalMetals project 'Recognition of microbial functional communities and assessment of the mineralizing potential (bioleaching) for high-tech critical metals' (https://www.uc.pt/en/org/biocriticalmetals, accessed on 20 July 2022). The Los Cóndores mine has a relatively long mining history from late 1800 to 1985 by exploitation of wolfram, bismuth, and copper ore deposits [14,15].

In October and November 2016, different kinds of soil samples and mine wastes were collected (approximately 2 kg each), but for this study, only four samples were used from two tailings and two dumps of the Los Cóndores mine (Figure 1; Supplementary Table S1).

The quartering and homogenization corresponding to the four samples were carried out in the mineral processing laboratory at Universidad Nacional de San Luis (Argentina). Subsamples were analyzed by Total X-Ray Fluorescence (TXRF), and the remaining samples were kept in the Bioenvironmental Laboratory (CNEA FRSR-UTN San Rafael, Mendoza province, Argentina) until their use in the bioreactors.

Figure 1. Geographical location of the Los Cóndores Mine in San Luis province (Argentina). Black points indicate the sampling sites under study.

2.2. Mycorrhizal-Assisted Phytomining (MAP) System

The MAP system consisted of sunflower plants (*Helianthus annuus* L., hybrid cultivar DK4045, Syngenta seeds) colonized by the AM fungus *Rhizophagus intraradices* GA5 strain grown in pots (1.2 L) with a homogeneous mixture of contaminated mine soil substrate (CS) or blank soil substrate (B), mixed with volcanic ashes (50:50, v/v) and supplemented with $ZnSO_4$ (as a catalyst). The B treatment consisted of commercial soil substrate (Fertile Soil Arhumus®).

The AM fungus GA5 strain, provided by Banco de Glomeromycota in vitro (Faculty of Exact and Natural Sciences of Buenos Aires University, Argentina, https://bgiv.com.ar/strains/Rhizophagus-intraradices/ga5, accesed on 20 July 2022) was propagated as described in Silvani et al. [16]. Four sunflower plants from each pot with the CS and B substrate were inoculated with 5 gr of general inocula of GA5 strain (containing mycorrhizal root fragments, external spores, and mycelia). The other four sunflower plants remained uninoculated with the AM fungus. The MAP systems were maintained for 30 days at controlled temperature (23 C day/16 C night) and natural light conditions until transplantation to the bioreactors.

2.3. Bioreactors at TRL 4

Two bioreactors, placed in the Bioenvironmental Laboratory (CNEA FRSR, Mendoza), were made with polycarbonate containers (30 cm × 60 cm and 80 cm in height and with a slope of 6%) (Figure 2) [13]. Each bioreactor was filled with three layers of stones of different granulometry. The first deeper 10 cm layer consisted of coarse gravel (average diameter = 10 cm), then a second 15 cm layer of medium gravel (average diameter = 5 cm), a third 20 cm layer of fine gravel (average diameter = 1 cm), and the upper 15 cm layer was composed of mine soil samples, volcanic ashes 1:1 (v/v) and 350 ppm $ZnSO_4$ (mine treatment, CS) (Figure 2). The control treatment was performed with the commercial soil substrate (blank soil treatment, B). Each container was connected to a collection chamber (10 cm × 30 cm and 80 cm deep) to collect percolated water. These bioreactors at TRL 4 were made to a 1:10 scale with the vegetable depuration module (VDM) at TRL 6 [8], taking into account the dimensions, slope, filter layers, and physicochemical (pH, Eh) and hydraulic parameters (hydraulic constant, hydraulic retention time, and vertical income flow).
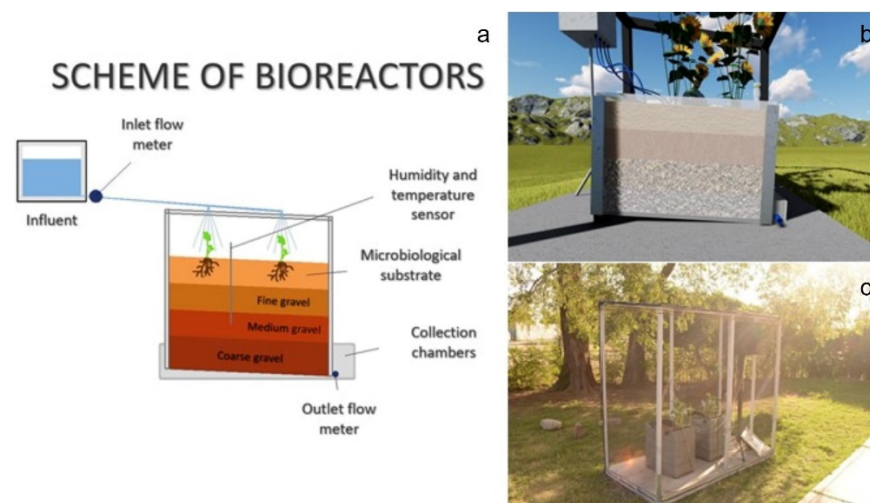
Figure 2. Schemes of the bioreactor (TRL 4). Transversal section of the bioreactor (**a**,**b**) and general view of the bioreactor at the Bioenvironmental Laboratory (**c**).

2.4. Hydraulic Calibration

The bioreactors and the VDM behave as a modified subsurface artificial wetland with inlet vertical flow and outlet flow collected in a collecting chamber (Figure 2). The volume of effluent obtained in the collecting chamber was not significant, given that irrigation along the assay was carried out considering the humidity of the substrate registered with sensors immersed into the upper top layer (CS layer).

To model the hydraulic flow in the bioreactors and the VDM, Darcy's law was used [13]. This law considers the inlet and outlet flow when field capacity is reached. The hydraulic constant (Ks) was also estimated. The hydraulics in both followed the Darcy model:

$$Ks = Q/(Ac \times s) \tag{1}$$

where:

Ks: hydraulic constant (m/days);
Q: average flow rate at which it enters and which it exits (m^3/days);
Ac = perpendicular area to the flow (m^2) = 0.3 m $\times$ 0.5 m;
s = slope (m/m) = 0.03 m/0.5 m.

Furthermore, the hydraulic retention time (th) was measured by registering the time it took the influent to cross down the different layers and to exit towards the collecting chamber when a 2 cm film of the fluent covered the last surface layer of stone in the bioreactor.

2.5. Experimental Design and Measurement of Parameters

Two bioreactors were performed as described above under two treatments: blank soil (B) and contaminated mine soil substrate (CS). In each bioreactor, 8 sunflower plants in total were grown: 4 plants were inoculated with the AM fungus GA5 strain and 4 were not inoculated. The plants were contained in separate pots in each bioreactor to avoid AM root colonization in uninoculated treatments. Previously, a complete hole in the bottom of each pot was made to let drainage occur. After 6 months, sunflower plants were harvested from each bioreactor. The shoots were separated into leaves and flowers, and the roots were carefully rinsed with distilled water to eliminate substrate particles. Fresh weights of leaves, flowers, and roots were registered, then dried in an oven at 80 °C for 72 h to obtain dry biomass.

To assess the mycorrhizal colonization, subsamples of roots were stained with trypan blue following the modified technique of Phillips and Hayman [17]. The frequency of mycorrhizal colonization was calculated as the percentage of root segments containing

AM fungal structures according to Giovanneti and Mosse [18]. All measurements were performed under a Nikon Optiphot-2 light binocular microscope at $100\times$ magnification. Finally, the chemical elements of dried plant tissues (roots, leaves, and flowers) and soil substrate from pots were analyzed by the Total X-Ray Fluorescence (TRX) technique. The bioconcentration factors (calculated as the ratio between the concentration of chemical elements in plant tissue and soil substrate, BC) and translocation factors (TF, calculated as the ratio between the concentration of chemical elements in leaves and roots and between leaves and flowers) were determined for the following chemical elements: Cr, Zn, P, Ni, Rb, Sr, Cu, and Mn [8,12].

2.6. TXRF Analysis

The chemical determination of plant biomass and soil substrate from each pot was carried out by the TXRF analysis. For that, each plant sample was weighed and placed in a Teflon beaker with 3 mL sub-boiling HNO_3 and 1 mL H_2O_2, and then microwave digested. After digestion, the solution was transferred to a 10 mL volumetric flask (or 5 mL in cases where the sample was smaller than 0.05 g) and made up to volume with Milli-Q water. A total of 10 μL of Ga 100 mg/L (Merck, Munich, Germany) was added as an internal standard to 1 mL of sample solution. Then, μ5 L of the solution was deposited on a quartz reflector and measured for 300 s in the TXRFS2 Picofox spectrometer (Bruker, Buenos Aires, Argentina) with a Mo tube. For soil samples, a 28 mm diameter pressed pellet was prepared with 3 g of dry sample. Elemental determination was carried out with a WDXRF S8 Tiger spectrometer (Bruker, Munro, Buenos Aires, Argentina), using the Quant Express application.

2.7. Bioextracting Potential (BP) in VDM

The estimation of BP of CRMs (P and Sr) and SRMs (Cr, Cu, Mn, Zn, Rb, and Ni) by using the MAP system in the VDM at TRL 6 containing the same contaminated mine soil, was carried out as detailed in Scotti et al. [5]. The VDM is located at the Bioenvironmental Laboratory CNEA FRSR (San Rafael, Mendoza, Argentina) and a description of its performance is detailed in Scotti et al. [8]. The estimation of BP of SRMs and CRMs by the VDM and the MAP system was calculated considering the concentration of each element in plant biomass obtained at the TRL 4 experiment, and the total biomass grown in the VDM with 1 m^3 of contaminated mine soil substrate, as follows:

$$BP \text{ (mg)} = [(Wtot \text{ (L)} \times Cp(L)) + (Wtot \text{ (R)} \times Cp(R)) + (Wtot \text{ (F)} \times Cp(F))]/1000 \quad (2)$$

where:

Wtot (L, R, F): Total dry weight (g) of aerial (leaves, L and flowers, F) and radical (roots, R) plant tissue;

Cp (L, R, F): concentration (ppm) of chemical elements in the aerial (Leaves, L and flowers, F) or radicular (roots, R) plant tissue.

3. Results

After calibration, we registered a flow rate (Q) of 7.3 m^3/day and the hydraulic retention time resulted in 10 min. After application of the Darcy's law equation, we obtained a hydraulic constant value (Ks) of 811.11 m/day for both bioreactors.

At the end of the experiment, all sunflower plants had survived, and no visible signs of toxicity were observed during the assay in the bioreactor. The dry biomass of sunflower plants grown in the contaminated mine soil substrate (CS) and blank soil (B), inoculated (M+) and uninoculated (M−) with the AM fungus Rhizophagus intraradices GA5 strain after 6 months is shown in Table 1. No statistical variation in plant biomass was registered between M+ and M− in both B and CS substrates. The root biomass from the CS treatment was lower than those values from the B treatment, whilst the leaf biomass of mycorrhized plants growing in the CS substrate had more biomass than those uninoculated plants.

Table 1. Plant dry biomass of Helianthus annuus grown in the contaminated mine soil substrate (CS) and blank soil (B) and inoculated (M+) and uninoculated (M−) with the arbuscular mycorrhizal fungus Rhizophagus intraradices GA5 strain.

		Biomass (g)	
Soil Substrate	**Plant Tissue**	**M+**	**M−**
CS	F	0.43 (0.02)	0.40 (0.03)
	L	0.29 (0.03)	0.20 (0.02)
	R	0.02 (0.01)	0.010 (0.006)
B	F	0.39 (0.03)	0.41 (0.03)
	L	0.31 (0.03)	0.30 (0.02)
	R	0.16 (0.01)	0.17 (0.01)

The Fisher test did not show significant differences between M+ and M− in B and CS treatments. Values are mean and standard deviation is reported in brackets (n = 4). F: Flower tissue; L: Leaf tissues; R: Root tissues.

The AM fungus R. intraradices GA5 strain colonized the sunflower roots in both substrates after 6 months (Figure 3a,b), but the percentage (%) of mycorrhizal root colonization of sunflowers grown in the CSM+ treatment was 45 ± 7.5 % (mean $\pm$ error standard), while the uninoculated plants showed a 3 ± 0.5 % in the same substrate (CSM−). The inoculated plants in the B treatment reached 88 ± 3 % of root colonization (BM+), and no colonization of roots by any AM fungi was observed in the roots of sunflowers uninoculated and developed in the same substrate (Figure 3c).
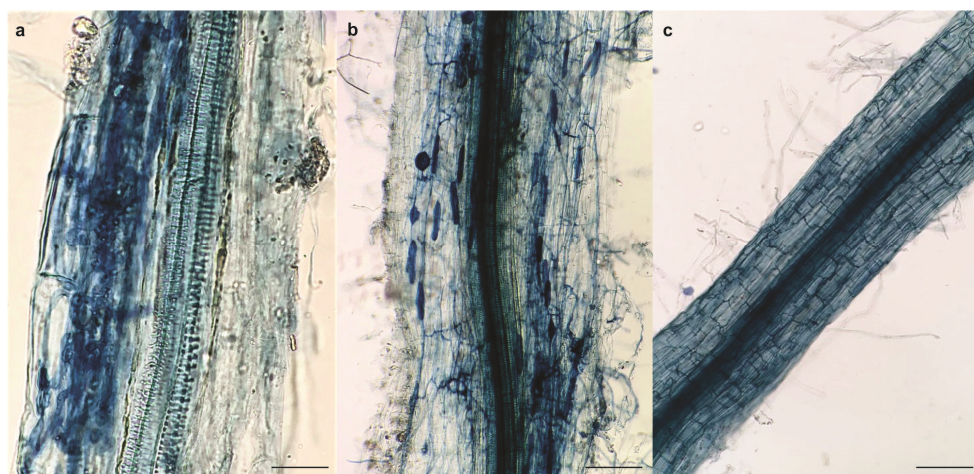

Figure 3. (**a**) Sunflower root fragments colonized by the arbuscular mycorrhizal fungus *Rhizophagus intraradices* GA5 strain after 6 months of growth in contaminated mine soil + volcanic ash + Zn (CSM+), and (**b**) in blank substrate (BM+); and (**c**) uncolonized root fragment of sunflower plant without inoculation of AM fungi and grown blank substrate (BM-). Bars: (**a**) 100 μm; (**b**) 200 μm; (**c**) 400 μm.

Table 2 shows the concentration of the raw materials (ppm) in different plant tissues of sunflowers inoculated (CSM+) and uninoculated (CSM−) with the AM fungus when grown in the CS and B substrate. The concentration of Zn, Cu, and Rb in the CS was markedly superior to the B substrate, while the P concentration resulted lower in the mining substrate. No differences were recorded between inoculated and uninoculated treatments for these elements. Zn accumulated more in the different plant tissues in both CSM− and CSM+ treatments. For Cr, no statistical differences were found among treatments, but a slight increase was observed in flower and leaves tissues and a decrease in roots when sunflowers were inoculated and developed in the mining substrate. In addition, more concentration of Cr was registered in the mining substrate when AM fungus was present. The P concentration was markedly different in B and CMS+ treatment in leaves of

sunflowers. For Ni, a higher concentration was found in the leaves and roots of inoculated plants grown in a contaminated substrate. No statistical differences were found in Cu concentration between CSM− and CSM− treatment but a tendency of increasing Cu in leaves and roots of sunflowers under CSM+ treatment was observed. Mn concentration was higher in the leaves and flower tissues of plants under CS treatment but without statistical differences between inoculated and uninoculated plants. For Rb and Sr, no significant differences were found between treatments. However, the Rb concentration in aerial parts of sunflower plants was slightly higher in the CSM+ treatment, and more Sr was concentrated in leaves with a decrease in roots in the CSM− treatment.

Table 2. Raw materials concentration (ppm) in the different plant tissues of *Helianthus annuus* inoculated (CSM+) and uninoculated (CSM−) with the arbuscular mycorrhizal fungus *Rhizophagus intraradices* GA5 strain, when grown in contaminated mine soil + volcanic ash + Zn (CS) and control blank soil (B).

Chemical Elements	Plant Tissues and Soil	B	CSM−	CSM+
Zn	Flowers	46 (4.24) **a**	756 (76) **b**	600 (60) **b**
	Leaves	41 (0) **a**	1200 (100) **b**	930 (93) **b**
	Roots	38.5 (12.0) **a**	1200 (100) **b**	1100 (100) **b**
	Substrate	137.5 (2.1) **a**	6000 (90) **b**	5900 (90) **b**
Cr	Flowers	3.15 (0.64) **a**	3.50 (0.70) **a**	4.02 (0.80) **a**
	Leaves	5.4 (0.6) **a**	4.7 (0.70) **a**	7.4 (0.7) **a**
	Roots	17.9 (14.2) **a**	29 (3) **a**	13 (1) **a**
	Substrate	64 (1) **a**	83 (8) **a**	95 (19) **a**
P	Flowers	3250 (353) **a**	1700 (200) **b**	1500 (200) **b**
	Leaves	2050 (353) **a**	615 (61) **b**	1500 (200) **a**
	Roots	1250 (212) **a**	584 (58) **b**	543 (54) **b**
	Substrate	3350 (70) **a**	2200 (300) **b**	2200 (300) **b**
Ni	Flowers	0.5 (0) **a**	0.7 (0.1) **a**	0.5 (0.1) **a**
	Leaves	0.5 (0) **a**	0.80 (0.02) **b**	1.4 (0.02) **c**
	Roots	1.75 (0.92) **a**	2.2 (0.3) **a**	5.2 (0.5) **b**
	Substrate	30.5 (0.7) **a**	33 (3) **a**	31 (6)a
Cu	Flowers	5 (0.7) **a**	15 (1) **b**	15 (1) **b**
	Leaves	8.4 (0.6) **a**	19 (2) **b**	25 (3) **b**
	Roots	11.25 (3.61) **a**	244 (24) **b**	254 (25) **b**
	Substrate	51.00 (1.41) **a**	4500 (700) **b**	4600 (700) **b**
Mn	Flowers	19.5 (3.5) **a**	118 (12) **b**	120 (12) **b**
	Leaves	32.5 (0.7) **a**	241 (24) **b**	338 (34) **b**
	Roots	51.5 (19.1) **a**	60 (6) **a**	84 (8) **a**
	Substrate	690 (19) **a**	1000 (200) **a**	1000 (200) **a**
Rb	Flowers	12.5 (0.7) **a**	15 (2) **a**	16 (2) **a**
	Leaves	10.7 (1.8) **a**	16 (2) **a**	16 (2) **a**
	Roots	21 (3) **a**	12 (1) **b**	14 (1) **ab**
	Substrate	82 (58) **a**	283 (28) **b**	280 (56) **b**
Sr	Flowers	95.5 (7.8) **a**	98 (10) **a**	97 (10) **a**
	Leaves	156.5 (6.4) **a**	220 (22) **a**	208 (21) **a**
	Roots	123.5 (13.4) **a**	93 (9) **a**	103 (10) **a**
	Substrate	138.5 (31.8) **a**	181 (18) **a**	177 (35) **a**

LSD Fisher—different letters in the rows indicate significant differences. The standard deviation is reported in brackets.

Table 3 shows the bioaccumulation coefficient (BC) values obtained in the different tissues of sunflower plants inoculated with the AM fungus *R. intraradices* GA5 strain and grown under contaminated mine substrate (CSM+). The BC values for flower biomass were 0.003 Cu < 0.016 Ni < 0.04 Cr < 0.06 Rb < 0.10 Zn < 0.12 Mn < 0.55 Sr < 0.68 P, for leaf biomass covered the range of 0.005 Cu < 0.045 Ni < 0.06 Rb < 0.08 Cr < 0.16 Zn < 0.34 Mn <

0.68 P < 1.17 Sr, and for root biomass resulted in 0.05 Rb < 0.06 Cu < 0.08 Mn < 0.14 Cr < 0.17 Ni < 0.19 Zn < 0.25 P < 0.58 Sr. The BC values in the CS treatment for flower biomass of sunflower plants without mycorrhizal inoculation (CSM-) were 0.003 Cu < 0.0021 Ni < 0.04 Cr < 0.05 Rb < 0.13 Zn < 0.12 Mn < 0.54 Sr < 0.77 P, whilst BC values for leaf biomass were 0.004 Cu < 0.024 Ni < 0.06 Cr = Rb < 0.20 Zn < 0.24 Mn < 0.27 P < 1.21 Sr, and for root biomass were 0.04 Rb < 0.05 Cu < 0.06 Mn < 0.07 Ni < 0.20 Zn < 0.26 P < 0.35 Cr < 0.51 Sr (Table 3). The BC values in the control treatment (B) for flower biomass resulted in 0.016 Ni < 0.03 Mn < 0.05 Cr < 0.08 Rb < 0.1 Cu < 0.33 Zn < 0.69 Sr < 0.97 P, for leaf biomass from 0.016 Ni < Mn < Cr < Rb < Cu < Zn < P< 1.13 Sr, while BC values for root covered the range 0.06 Ni < 0.07 Mn < 0.11 Rb < 0.22 Cu < 0.28 Cr = Zn < 0.37 P < 0.89 Sr (Table 3). The highest values of BC for P and Mn were registered in the aerial part when sunflower plants were inoculated and grown in the mine substrate (CSM+), while Sr showed BC > 1 without significant differences between treatments. Meanwhile, the highest values of BC were observed in roots for Cr, Cu, and Ni in comparison with leaves and flower tissues, and similar BC values were obtained for Zn in roots and leaf tissues of sunflower plants for treatments with the mine substrate (CSM+ and CSM−). The highest translocation factors (TF) (root to leaf) in mycorrhizal plants were recorded for Mn (4.02) > Sr > P > Rb > Zn (0.84) (Table 3). The mycorrhizal treatment decreased Cr input to the root but increased the TF for this element. Furthermore, mycorrhization increased the translocation of Cu, Cr, and P from root to leaf, but no variation occurred for Mn, and for Zn, Rb, Ni, and Sr the TF values were lower. In the case of translocation from leaf to flower tissues, the TF factor only increased for Rb in mycorrhizal treatments.

Table 3. Values of the bioaccumulation coefficients (BC) and translocation factors (TF) from roots to leaves and from leaves to flowers of the chemical elements under study in *Helianthus annuus* plants growing in blank soil (B) or contaminated soil substrate (CS), inoculated (CSM+) and uninoculated (CSM-) with the arbuscular mycorrhizal fungus *Rhizophagus intraradices* GA5 strain. The comparative effects of TF in mycorrhization and control treatment are shown as greater (+), less (−), or equal (=).

Elements	Treatment	BC Flower	BC Leaves	BC Roots	TF Leaves/Roots	Effect M+	TF Flower/Leaves	Effect M+
Cr	CSM+	0.04 (0.02)	0.08 (0.02)	0.14 (0.04)	0.57	(+)	0.50	(−)
	CSM−	0.04 (0.01)	0.06 (0.01)	0.35 (0.07)	0.16		0.67	
	B	0.05 (0.01)	0.09 (0.01)	0.28 (0.23)	0.30		0.57	
Zn	CSM+	0.10 (0.01)	0.16 (0.02)	0.19 (0.02)	0.84	(−)	0.63	(−)
	CSM−	0.13 (0.02)	0.20 (0.02)	0.20 (0.02)	1.00		0.65	
	B	0.33 (0.04)	0.30 (0.01)	0.28 (0.10)	1.06		1.10	
P	CSM+	0.68 (0.18)	0.68 (0.18)	0.25 (0.06)	2.76	(+)	1.00	(−)
	CSM−	0.77 (0.20)	0.28 (0.07)	0.26 (0.06)	1.05		2.75	
	B	0.97 (0.13)	0.61 (0.12)	0.37 (0.07)	1.64		1.59	
Cu	CSM+	0.003 (0.0007)	0.005 (0.001)	0.06 (0.01)	0.10	(+)	0.60	(−)
	CSM−	0.003 (0.0007)	0.004 (0.001)	0.05 (0.01)	0.08		0.75	
	B	0.10 (0.02)	0.16 (0.02)	0.22 (0.08)	0.75		0.63	
Mn	CSM+	0.12 (0.04)	0.34 (0.10)	0.08 (0.02)	4.02	(=)	0.35	(−)
	CSM−	0.12 (0.04)	0.24 (0.07)	0.06 (0.02)	4.02		0.50	
	B	0.03 (0.006)	0.05 (0.002)	0.07 (0.03)	0.63		0.60	
Rb	CSM+	0.06 (0.02)	0.06 (0.02)	0.05 (0.01)	1.14	(−)	1.00	(+)
	CSM−	0.05 (0.01)	0.06 (0.01)	0.04 (0.01)	1.33		0.83	
	B	0.15 (0.12)	0.13 (0.11)	0.26 (0.22)	0.51		1.15	
Sr	CSM+	0.55 (0.16)	1.17 (0.35)	0.58 (0.17)	2.02	(−)	0.47	(−)
	CSM−	0.54 (0.11)	1.21 (0.24)	0.51 (0.10)	2.37		0.45	
	B	0.69 (0.21)	1.13 (0.30)	0.89 (0.30)	1.26		0.61	
Ni	CSM+	0.016 (0.006)	0.045 (0.01)	0.17 (0.05)	0.27	(−)	0.36	(−)
	CSM−	0.021 (0.005)	0.024 (0.003)	0.07 (0.02)	0.36		0.88	
	B	0.016 (0.0004)	0.016 (0.0004)	0.06 (0.03)	0.29		1.00	

Standard deviation is reported in brackets.

Table 4 shows the estimation of bioextracting potential (BP) for each chemical element when using the MAP system in the VDM containing 2 m^3 of the substrate (50:50, *v/v* consisted of contaminated soil: volcanic ash). The BP (mg) varied in 0.21 Ni < 1.2 Cr < 3.4 Rb < 5.45 Cu < 30.2 Sr < 43.9 Mn < 159 Zn < 316 P. The highest BP estimated value considering root and leaf biomass was obtained for P, Zn, Mn, and Sr.

Table 4. Estimation of bioextracting potential (BP) for each chemical element when using the MAP system in the VDM containing 2 m^3 of substrate (50:50, *v/v* consisted of contaminated soil: volcanic ash).

Parameter	Zn (ppm)	Cr (ppm)	P (ppm)	Ni (ppm)	Cu (ppm)	Mn (ppm)	Rb (ppm)	Sr (ppm)
CpF × WtF	74,820 (10,962)	501 (123)	187,050 (33,640)	62.3 (15.4)	1870 (211)	14,964 (2192)	1995 (342)	12,096 (1810)
CpR × WtR	6380 (3770)	75 (43)	3149 (1888)	30 (18)	1473 (882)	487 (290)	81 (46)	597 (357)
CpL × WtL	78,213 (15,912)	622 (123)	126,150 (29,870)	118 (14)	2102 (470)	28,425 (5800)	1346 (307)	17,492 (3576)
BP (mg)	159 (30)	1.2 (0.3)	316 (65)	0.21 (0.05)	5.4 (1.6)	43.9 (8.3)	3.4 (0.7)	30.2 (11.8)

Standard deviation is reported in brackets. Cp (L, R, F): concentration (ppm) of chemical elements in leaves (L), flowers (F), or roots (R) tissues; Wtot (L, R, F): total dry weight (g) of leaves (L), flower (F) or roots (R) tissues.

4. Discussion

In this work, the mycorrhizal-assisted phytomining (MAP) system, composed of *Helianthus annuus*–arbuscular mycorrhizal fungus *Rhizophagus intraradices*–Zn-volcanic ashes was evaluated in bioreactors at TRL 4 for the recovery of CRMs (Sr, P) and SRMs (Cr, Zn, Cu, Mn, Rb, Ni) from Los Cóndores mining wastes. The mine substrate showed high concentrations of Cu, Zn, Rb, and low P concentration in comparison with the blank substrate and other mining-contaminated soils previously reported [2,5]. The variation in composition and concentration of chemical elements in the substrate might cause differences in the behavior of the MAP system. It is known that AM symbiosis functions as a 'buffer' to protect the host plant against damage caused by heavy metals in the soil [19,20]. The different behavior against heavy metals in excess may be due to competition for membrane receptors or passive diffusions under pH and Eh values in the experiment, as well as to the activation of certain fungal and/or plant enzymes/glycoproteins that are involved in chelation, speciation (redox processes), sequestration and immobilization of chemical elements (by phytochelatins, glomalin, or fungal metallothioneins) [19]. Some substances such as Zn ions act as enzyme cofactors that activate/deactivate enzymes involved in the tolerance and uptake/exclusion of chemical elements at high concentrations [8,11]. In this sense, we highlighted that the concentration of Zn in the MAP system affected the entry of different chemical elements in mycorrhizal plants. In addition, the inoculation with AM fungus *R. intraradices* GA5 strain favored phytoextraction processes by increasing translocation from roots to leaves of Cr, P, and Cu. On the other hand, there were no differences in Mn behavior, and phytostabilization occurred for Ni, Sr, Rb, and Zn by decreasing its translocation to aerial plant tissues. The same mycorrhizal effect on translocation from roots to leaves of Cr, Cu, and Ni, and the opposite mycorrhizal effect for Zn, P, Sr, Rb, and Mn were found with the MAP system developed in mine substrates from Odisha (India). These differences could be attributed to the different physicochemical characteristics of the mine substrates under study (Odisha and Los Cóndores mines) [2,5].

When mycorrhized, plants grown in the blank soil substrate increased the concentration of P in leaves, Ni in leaves and roots, and Rb in roots. An increase in the translocation of P from roots to leaves in M+ treatments was observed. It is widely known that AM fungi improve the nutritional status of colonized plants, mainly by P and N uptake [21], and also contribute to the enhancement of Ni and Cr in sunflower biomass [5,22].

No differences in Sr bioaccumulation were found between inoculated and uninoculated treatments, but a very significant difference was observed in the bioaccumulation of this element in leaves compared to roots (TF > 2). Many authors have confirmed that Sr can be accumulated in leaves, leaf trichomes, and stems in several plant species [2,5,23,24].

Rb also presented a higher bioconcentration value in leaves (with a TF > 1) of sunflower plants grown in the mine substrate in comparison with those in the blank soil substrate.

As in previous works, at considerable amounts of Rb in the substrate (>280 ppm) and after reaching a bioconcentration threshold in leaves, the mycorrhizal treatment exerts an exclusion system and slows down the translocation of Rb to the aerial plant tissues. This effect had not been observed in our previous works probably due to the low concentrations of Rb in a substrate. Rb ion is an analogue for K ion, which is taken up along the same pathways as K and typically occurs at very low levels in soils and plant tissues [25].

A low plant biomass production in the mining substrate was recorded. This result could be due to the high concentration of some toxic elements in the mine soil that affected sunflower plant development. Likewise, mycorrhizal root colonization is generally reduced at high heavy metal concentrations in soil, and the potential uptake of Zn and other nutrients may be reduced [26,27]. For this reason, it is possible that we have not found a significant difference in plant biomass and bioconcentration values for many metals between inoculated and uninoculated treatments.

We detected a decrease of translocation in most heavy metals from the leaves to the flower parts when plants were colonized by AM fungi, except for Rb (which did not present significant differences). De María and Rivelli [28] found differences in the accumulation and distribution of Cd, Zn, and Cu in diverse phenological stages; these metal concentrations increased in the stems and leaves, particularly in the old ones, whereas decreased in bud flowers. The storage of some heavy metals in roots and their low translocation along the plant tissues during the growth stage could be considered a strategy of mycorrhizal sunflower plants to preserve young metabolically active leaves and reproductive organs from toxic metal concentrations [28]. Our results demonstrate a promising biotechnology applicable to agronomical practices given the exclusion of toxic elements in flower parts.

The highest BP of the MAP system in the VDM at TRL 6 containing mine residues was for P, Zn, Mn, and Sr (considering root and leaves). These values are much lower than those found in Scotti et al. [5] and those obtained in Guglietta et al. [2] with the same MDV system. The different mine substrates could be the cause of performance variation of the MAP system in the VDM. For that reason, calibration of those variables in the bioreactor at TRL 4 is needed before scaling up at a higher TRL maturity. The BP encourages a recovery of SRMs and CRMs by hydrometallurgical techniques [1], with subsequent purification by selective electrodeposition, permitting a sustainable and selective metals recovery at a high degree of purity (95%) and determining commercial reuse. By application of a process circuit with leaching/purification of the SRMs and CRMs accumulated and concentrated on plant biomass, a recovery of 90-95% of purified metals has been demonstrated [1,29].

5. Conclusions

Interesting aspects in the behavior of the MAP system regarding the extraction, translocation, and exclusion of chemical elements were found, associated with the composition of the substrate.

The MAP system allowed the extraction of the elements under study, being tolerant to high concentrations of heavy metals.

A high efficiency of the MAP system for the exclusion of heavy metals in flower tissues was registered.

The BP was effective for Sr and P more than other elements. These results are of great importance given that P and Sr are valuable CRMs.

The results encourage the application of this methodology but improve plant biomass; this is a critical issue for the sustainable exploitation of mining waste within a circular economy context.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/met12111828/s1, Table S1. Additional information about sampling sites (Source Verónica Saavedra, Universidad Nacional de San Luis, Horizon 2020 ERA MIN BioCriticalMetals project).

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