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Biotechnological Tool for Metal(loid)s as Cd, Cu, Ni, and P Management with Multiple Approaches: Bioremediation, Recovery of Raw Materials, and Food Safety

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Abstract: Contaminated soils are a challenge for implementing biotechnology in bioremediation, the recovery of Critical and Strategic Raw Materials (CRMs and SRMs), and food security. European Union (EU) Governments have established strict limits on As, Pb, Cd, and Hg in foods (Document 32023R0915) and requested the recovery of 34 CRMs within a circular economy (CE) (5th CRMs list). This study proposed a biotechnological tool for the decontamination of soil with heavy metal(loid)s by arbuscular mycorrhizal (AM)-assisted phytoextraction and the subsequent recovery of CRMs or by phytostabilization to prevent their entry into the food chain. It consisted of placing *Baccharis salicifolia* plants, inoculated or non-inoculated with AM fungi, into bioreactors (BRs) containing mining soil with Cd, Ni, and Cu, according to the Argentinian Patent (AR090183B1). The bioextractive potential (BP) was also estimated at the highest Technological Readiness Level (TRL) using a vegetable depuration module (VDM, TRL 6). Inoculated plants showed significantly higher aerial bioaccumulation coefficients (Cd: 68.62; P: 2.99; Ni: 2.51; Cu: 0.18) in BRs, and the BP values reached 1.16 g, 9.75 g, 2.40 g, and 213.1 g for Ni, Cd, Cu, and P, respectively. Finally, these CRMs and SRMs could be recovered from biomass through hydrometallurgy within a CE framework.

Keywords: food security; circular economy; mycorrhizal-assisted phytomanagement; TRL 6; critical raw materials

1. Introduction

The elevated content of heavy metal(loid)s in soils, whether from natural sources or anthropogenic activities, presents a challenge for developing biotechnologies to manage these elements. Many of these polluting elements are considered Critical Raw Materials (CRMs) or Strategic Raw Materials (SRMs), which should be recovered within a circular economy (CE) model. The CE represents a completely new concept for the life cycle of a product, emphasizing sustainable activities such as recycling and reuse. This model is

gradually replacing the traditional linear economy of the 'take—make—dispose' model, as it offers significant environmental benefits through waste reduction and economic advantages by saving on Raw Materials (RMs) [1].

In general terms and without any intention of giving a definition of CRMs, a material may also be considered critical if it is a commodity produced exclusively in one country or region with unstable regimes. CRMs are very important to the worldwide economy and have a high risk associated with their supply. On the other hand, SRMs are any RMs vital to an individual's or organization's strategic planning and supply chain management. A shortage of these materials could leave an organization or government vulnerable to disruptions in the manufacturing of essential products. SRMs are crucial for making countries more self-sufficient in strategic products, especially in green energy areas, digital technology, and defense.

In 2023, the fifth list of 34 CRMs and SRMs was published in Annex II of the Regulation proposal COM (2023) based on the Study on the CRMs for the European Union (EU) 2023—Final Report. The materials listed are as follows (* indicates SRMs): bauxite, antimony, arsenic baryte, beryllium, bismuth *, boron */borate, cobalt *, coking coal, feldspar, fluorspar, gallium *, germanium *, hafnium, helium, heavy rare earth elements *, lithium *, light rare earth elements *, manganese *, natural graphite *, niobium, platinum group metals *, phosphate rock, copper *, phosphorus, scandium, silicon metal *, strontium, tantalum, titanium metal *, tungsten *, vanadium, nickel *, and magnesium * [2]. In this context, it is essential to identify both natural and anthropic sources of CRMs and SRMs to address their potential recovery (Table 1).

Anthropogenic activities can also alter the moisture and temperature regimes of soils and groundwater, as well as increase the rates of movement of contaminants through soil erosion (by wind or water), soil runoff, leaching, and volatilization. For instance, dust storms, volcanic eruptions, geothermal hydrothermal activity, and forest fires can elevate the levels of As and Hg in the environment. The elevated content of heavy metal(loid)s in soils, whether from natural sources or anthropogenic activities; the insufficient documentation of contaminated areas in many regions; and the lack of regulations for their remediation further accentuate this environmental issue [3].

According to the AMI 2030 [4], while the EU excels in advanced materials and manufacturing, it risks falling behind in key future competitiveness areas such as Artificial Intelligence, Big Data, cloud computing, industrial biotech, robotics, and micro-electronics. The EU's technological capacities are insufficient for growth in key sectors, like Information and Communications Technology, biotechnology, energy, aerospace, and defense. Furthermore, the EU's research and innovation capabilities are trailing behind those of the United States of America and have been surpassed by China, which may lead to future dependencies on these technologies [5] (Figure 1).

Dinh et al. [6] highlighted the potential of phytomining for extracting noble metals within the CE model and the extraction of valuable chemical elements from subeconomic deposits or mineral wastes in plant biomass, which can be subsequently recovered. Phytomining is currently applied to the recovery of CRMs and SRMs from solid metal wastes, resulting in a promising option for advancing the CE.

On the other hand, if some of these elements (As, Cu, Ni, Cd, Be, Sb) enter the food chain, whether from land or water resources, in concentrations exceeding allowable limits, they can have toxic effects on human health in the short, medium, or long term [7,8]. Currently, there are several regions where the concentrations of these elements are regulated by several pronouncements. In this sense, the EU, in its document 32023R0915, establishes maximum limits for As, Pb, Cd, and Hg in food [9]. Food products from regions with these elements will be controlled and may be rejected for sale. In South American countries, high soil Cd concentrations, from volcanic eruptions and chemical fertilization, have contaminated cocoa beans, affecting their international marketability. Many attempts have been proposed to address this problem, and the Fontagro project (ATN/RF 18951-

RG) 'Bioprocess that reduces Cd rhizospheric solubility' specifically tackles this issue by implementing mycorrhizal-assisted phytomanagement (MAPm) [10].

Table 1. Natural and anthropic sources of some elements and their industrial use.

Element	Essential	Natural Sources	Anthropogenic Sources	Uses
Arsenic	No	Dust storms. Volcanic eruptions. Geothermal/hydrothermal activity. Forest fires. Arsenic-rich minerals.	Metal mining and smelting. Coal mining and burning of arsenic-rich coals. Pesticides. Timber industry. Pyrotechnics.	Wood preservatives. Additive to veterinarian drugs (poultry). Doping agent in semiconductors.
Cadmium	No	Zinc and lead minerals. Phosphate rocks.	Electroplating. Metal industry (nonferrous metals and steel). Automobile exhaust. Phosphate mineral fertilizers.	Pigments in paints, ceramics, plastics, etc. Cd impurities in Zn coatings used on metal structures (sacrificial coatings)
Chromium	Yes	Chromium minerals.	Metal industry. Electroplating. Industrial sewage.	Electroplating. Metal alloys. Anticorrosive products. Pesticides, detergents.
Copper	Yes	Sulfides, oxides, carbonates.	Domestic and industrial waste, mining waste, and manure (pig and poultry). Car brakes. Metal industry. Copper-based fungicides.	Electric supplies, electric conductors. Electroplating. Fungicides. Plant residues treated with fungicides are used as soil amendments. Timber treatment chemicals. Copper piping and guttering. Vehicle brake linings.
Lead	No	Lead minerals	Battery manufacturing facilities. Private and industrial waste. Rifle ranges and military facilities. Leaded paints and leaded fuel addition. Insecticides.	Batteries. Alloys, bullets, and other munitions.
Mercury	No	Mercury sulfide ores. Volcanoes. Forest fires. Ocean emissions	Artisanal and small-scale gold mining. Chemical industry. Fossil fuel (coal and petroleum) combustion. Nonferrous metal production.	Catalysts, electrical switches. Batteries, fluorescent lights, felt production, thermometers, and barometers. Alloys for dental fillings. Bright-red paint pigments.
Nickel	Yes	Nickel minerals.	Metalworks, battery plants, electronics. Industrial waste.	Metal alloys, batteries, electronics.
Zinc	Yes	Minerals.	Battery plants. Metal industry. Phosphate fertilizers.	Batteries. Alloys. Construction anticorrosive planting. Tire rubber. Additives in veterinary drugs and pesticides.

In some cases, the situation is so severe that many contaminated agricultural soils can no longer be used for food crops. However, lignocellulosic energy crops, which can grow on such soils, offer an alternative by producing biomass for bio-based materials and biofuels, thereby reducing the pressure on limited arable lands [11]. Nevertheless, this solution for using soil does not contribute to soil decontamination for the food industry unless the species used to obtain biomass for biofuel extract or reduce the bioavailability of the contaminants [12]. In previous works, we proposed the extraction of metals from mining waste from biomass derived from extractive plant species using hydrometallurgy [13] given that Green Supply Chain Management (GSCM) and sustainable waste management (SWM) are two pillars of the CE framework. To reduce the negative effects on the environment

caused by supply chains, GSCM employs sustainable materials, optimizes logistics, and uses energy-efficient techniques [14]. In this sense, we propose soil restoration techniques with low-cost biological systems that can be applied to mining waste and arable soils in the context of a CE. It is known that the costs of phytoremediation are low in relation to other methods used. Conventional physical and chemical methods are usually expensive and can irreversibly affect the properties of soil, water, and the living beings that inhabit them. Physical remediation methods include soil replacement, soil isolation, vitrification, and electrokinetics, while chemical methods include immobilization and soil washing. Biological and chemical methods can be applied together depending on the type of contaminant, soil, plant, and chemical reagent, with biological methods being more economical and eco-sustainable [3].

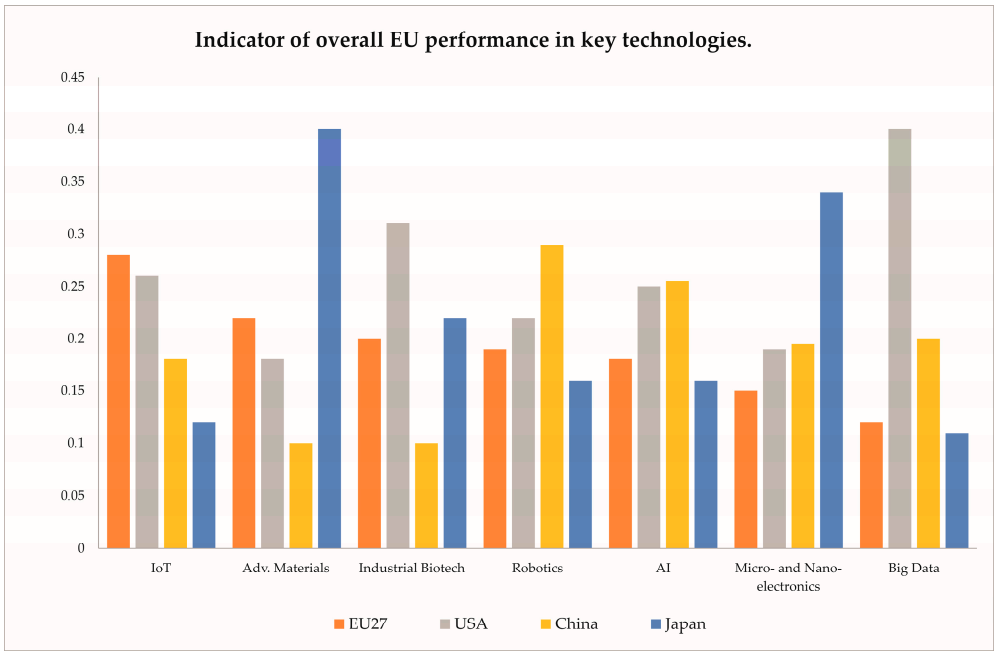

Figure 1. Indicators of overall European Union performance in key technologies. Adapted from Advanced Technologies for Industry [4,5].

MAPm is an innovative strategy for soil decontamination and sustainable waste management that utilizes mycorrhizal plants associated with specific arbuscular mycorrhizal (AM) fungi and advanced technology to achieve determined purposes such as (a) the phytoextraction and subsequent recovery of CRMs and SRMs from plant biomass; (b) the remediation of contaminated soils through the phytostabilization of heavy metal(loid)s in the matrix soil, thus mitigating their translocation to the aerial plant biomass or mobilization to other areas; and (c) the phytoextraction of toxic elements from areas already destined for agriculture production. Furthermore, the use of MAPm contributes to (a) carbon capture by increasing plant biomass and sequestration in soil C-pools through the accumulation of resistant glycoproteins released by AM fungi (glomalin); (b) the reduction in chemical fertilization by increasing soil phosphorus (P) availability and translocation to plants; and (c) the improvement in resistance to stress conditions (drought, salinity, and heavy metal(loid) soil contaminants), thus making it a valuable tool in the context of climate change [13–15].

The MAPm technology was substantially tested at laboratory scale (TRL 2–3) and can be easily scaled from TRL 4 to TRL 6 and further developed to become a protocol applicable in the territory (TRL 7), feasible to be used in the biotechnology industry in several approaches: 1—in the management of mining waste, either by phytostabilization or phytoextraction; 2—in in situ phytomining with the recovery of the mineral through hydrometallurgy; 3—in circular economy models since the mineral is recovered after the

treatment of the biomass, reducing the residue to its maximum expression since it is the residue of hydrometallurgy that finally remains; and 4—in food safety to prevent the contaminant from entering edible plant parts. Therefore, our hypothesis is that MAPm is an effective green biotechnology tool to improve food safety and the phytoextraction of CRMs and SRMs.

The aim of this work was to develop a biotechnological tool capable of decontaminating soils with high levels of heavy metal(loid)s, phytostabilizing them to prevent entry into the food chain, or phytoextracting them and recovering valuable chemical elements such as CRMs and SRMs. For this, we placed *Baccharis salicifolia (Ruiz & Pav.) Pers* plants, either inoculated or not with a mixture of native AM fungal species isolated from the *Paramillos de Uspallata* Zn-Pb-Ag mine, into bioreactors (BRs) containing either non-contaminated (blank) or contaminated soil with Cd, Ni, and Cu, following the procedure detailed in Argentina Patent No. AR090183B1 [16]. Additionally, the bioextractive potential (BP) of this biotechnological tool applied in the BRs (TRL 4) was estimated at the highest TRL by using a vegetable depuration module (VDM) (TRL 6). A glossary with biological definitions was added as Appendix A.

2. Materials and Methods

2.1. Mycorrhizal-Assisted Phytomanagement (MAPm) System

The MAPm system consisted of *B. salicifolia* plants, a native shrub found in the Complejo Minero Fabril San Rafael (CMFSR), a uraniferous mine belonging to the National Atomic Energy Commission (San Rafael, Mendoza province, Argentina), that was colonized by a mixture of AM fungal species, previously isolated from *Paramillos de Uspallata* mine (Mendoza, Argentina).

The AM fungal inoculum, composed of external mycelium, spores, and colonized root fragments, was obtained by culturing *Medicago sativa* (L.) as host plants in pots containing a sterilized substrate (soil/perlite, 2:1, *v*/*v*) for four months under greenhouse conditions. After this period, plants and the growth substrate were left to dry and checked for sporulation. The AM fungi were maintained by Banco de Glomeromycota in vitro (FCEN UBA, https://bgiv.com.ar/ accessed on 19 August 2024). *B. salicifolia* plants were grown in a greenhouse under natural light conditions for 6 months as detailed in Castaño et al. (2023) [13]. Then, plants were transplanted in the BRs and inoculated or not with the AM fungi, under experimental conditions.

2.2. Scale Up of TRL 6, Bioreactors (TRL 4), and Vegetable Depuration Module (VDM)

2.2.1. Experiment in Bioreactors

Four BRs were built in the Bioenvironmental Laboratory (CNEA FRSR, Mendoza Argentina) according to the procedure described in [13] (Figure 2a). Each BR was filled with three layers of stones of varying granulometry and a soil layer, and a chamber was used for planting (Figure 2b). Additionally, a collection chamber with a 6% slope was included to facilitate the flow of the leached solution from each chamber. In two BRs, the top 15 cm layer consisted of CMFSR mine soil supplemented with Cu (250 ppm as $CuSO_4$), Ni (7.5 ppm as $NiCl_2$), and Cd (2.60 ppm as $CdCl_2$) (contaminated treatment, CS), while in the other two BRs, the top 15 cm layer consisted of a blank soil (BS treatment) collected from a non-contaminated area from San Rafael (Mendoza). The preparation of the upper soil layer in the BRs, whether containing contaminants or not, followed the protocol outlined in [13] and the Argentinian patent [16]. The chosen concentration of Cd, Cu, and Ni was governed by those found in the soil of cocoa plantations in areas of Ecuador for Cd and in the soil of the natural wetland located in the Pilcaniyeu Technological Complex of the National Atomic Energy Commission, Argentina, for Cu and Ni.

Figure 2. The mycorrhizal-assisted phytomanagement (MAPm) system in bioreactors (BRs) (**a**–**c**) and the vegetable depuration module (VDM) (**d**–**f**) using *Baccharis salicifolia* plants and AM fungi. (**a**) BRs with control and contaminated soil; (**b**) transplant in a BR; (**c**) *B. salicifolia* in a BR after 4 months; (**d**) VDM view; (**e**) VDM hydraulic system view; (**f**) the MAPm system in the VDM.

Twelve 6-month-old *Baccharis salicifolia* plants were grown in each BR. Two BRs contained plants inoculated with 5.0 g of inoculum (M+), and the other two BRs were non-inoculated (M−) (Figure 2b,c). For mycorrhization, a 10 cm hole was made in the soil, and the AM inoculum was applied in the vicinity of the roots of *B. salicifolia* (Figure 2b). The MAPm system in the BRs was maintained for four months under natural environmental conditions and was irrigated with water every five days. The leached solution was collected from the collection chamber at the end of the experiment. The experiment had a 2 × 2 factorial design, with four BRs and four treatments: inoculated plants in blank soil (M+ BS), non-inoculated plants in blank soil (M− BS), inoculated plants in contaminated mine soil (M+ CS), and non-inoculated plants in contaminated mine soil (M− CS). After four months, the plants from each BR were harvested. The shoots were separated into leaves and stems, and the roots were carefully rinsed with distilled water to remove substrate particles. The fresh weight of the shoots and roots was recorded; then, they were dried in an oven at 70 °C for 48 h until constant weight to obtain dry biomass.

2.2.2. Potential Scaling Up of BRs to Vegetable Depuration Module (VDM)

The VDM is a technological development at TRL 6 in which the MAPm system was previously operated before to obtain an application protocol in the territory (TRL 7) [17,18]. The VDM was built at the Bioenvironmental Laboratory (Figure 2d). The module consists of two pools connected to collection chambers through a hydraulic system (Figure 2e). Each pool has a width of 3 m, a length of 5.0 m, and a depth ranging from 0.6 m (inlet end, lower depth) to 0.9 m (outlet end, upper depth), resulting in a height difference (Dh) of 0.3 m and a slope (Dh/length) of 6%. The collection chamber has a length of 1 m, a width of 3 m, and a depth of 1 m. The VDM was environmentally isolated with a waterproofing system and a metallic net-covered greenhouse with a polyethylene anti-hail film. Water

runs through pipes that are connected to a reserve tank and a water pump that drives the vertical flow toward the pool (Figure 2e,f). The remaining fraction of percolated liquid is drained towards the collection chamber. A stone filter for filling the pools was used in the same way as in the BRs.

The last layer of the VDM contained the contaminated soil simulating the real environment, in the same way as that in the upper layer of the BR. *B. salicifolia* plants were inoculated with a mixture of AM fungal species from the Paramillos mine. Each pool is equivalent in volume to 100 BRs. Scaling up from the BR to the VDM (100BR: 1VDM) was performed.

2.2.3. Hydraulic Calibration

The following hydraulic parameters calibrated for the scaling up from TRL 4 (BR) to TRL 6 (VDM) were considered:

- Type of water entry in irrigation;
- Income flow (Qi);
- Egress flow (Qe);
- Average Qi and Qe (Q);
- Liquid retention time before the exit to the collecting chamber (tc);
- The permeability constant (Ks) of the stone filter and soil substrate;
- The volume of the soil substrate (last layer with the soil);
- Stone filter volume;
- Water holding capacity (WHC);
- Porosity (φ);
- Darcy's velocity (V_D);
- Average linear velocity (Va).

The type of liquid entry in the calibration was vertical and laminar; thus, Darcy's law was applied to determine Ks, according to the following formula:

$$Ks = Q/(Ac \times s), \quad (1)$$

$$V_D = Q/Ac, \quad (2)$$

where Ks: permeability hydraulic constant (m/days); Q: average flow rate as (inlet flow + outlet flow)/2 (m^3/day); Ac = perpendicular area to the flow (m^2); s = slope (m/m); V_D = Darcy's velocity (Darcy flux).

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

It is important to note that Darcy flux does not equal the fluid velocity, even though it is expressed in units of velocity. The fluid velocity is found through the average linear velocity (Va), which is the average of the velocity of all possible fluid paths through the porous media

$$Va = V_D/\varphi, \quad (3)$$

where φ is the porosity, and V is the fluid velocity. The porosity, φ, is calculated as the ratio of the volume of void space to the total volume of a material, and is usually expressed as a fraction between 0 and 1 or as a percent.

$$\varphi = V_{void}/V_{tot} \quad (4)$$

Effective porosity is calculated as the volume collected in the collection chamber (V_{void}) divided by the total volume entered into the BR (V_{tot}).

Additionally, the water holding capacity (WHC) of the BR was determined by the following:

$$WHC = Mt - Ms, \quad (5)$$

where WHC is the mass of the water in kilograms, Mt is the total mass of the container and wet soil in kilograms, and Ms is the total mass of the container and dry soil in grams.

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

The parameters mentioned above must respect the relationship between the BR and VDM, maintaining a volume ratio of 100:1 and a surface ratio of 10:1, while ensuring that the permeability constant (Ks) remains similar in both the BR and the VDM.

2.3. AM Fungal Parameters

To determine AM fungal colonization in *B. salicifolia* plants, a subsample of roots was taken from each plant, cleared in KOH (10% *w*/*v*) for 15 min at 90 °C, rinsed in 5% HCl for 5 min, and stained with Trypan blue in lactic acid (0.02%) for 10 min at 90 °C. The frequency (% F) and intensity (% I) of mycorrhizal colonization were measured according to Declerck et al. [19]. Fifty randomly selected root pieces (1 cm length) were mounted on microscope slides in groups of ten and examined with an Olympus BX51 microscope at 400× magnification. % F was calculated as the percentage of root segments containing any intraradical AM structure (hyphae, arbuscules, coils, or vesicles), and % I was estimated by sorting out the root segments in different intensity classes (1–20%, 21–40%, 41–60%, 61–80%, and 81–100%), and the result was expressed as a percentage as follows: 10v + 30w + 50x + 70y + 90z)/(v + w + x + y + z), where v, w, x, y, and z are the number of roots in each class.

In addition, easily extractable glomalin-related soil proteins (EE-GRSPs) and total glomalin-related soil proteins (T-GRSPs) were measured, given the role of glomalin in the C sequestration and immobilization of heavy metal(loid)s in soils. The EE-GRSP fraction is composed of the newly produced or readily decomposed GRSP in soil, whilst T-GRSP is considered the recalcitrant fraction [15]. To determine the EE-GRSP and T-GRSP fractions, samples of soil (CS and BS) in each BR were harvested from the rhizosphere of plants, sealed in polyethylene bags, and stored at 4 °C until processing. The concentration of EE-GRSP and T-GRSP for each treatment was estimated according to Li et al. [20]. For EE-GRSP extraction, a sample of air-dried soil (1 g), taken from each plant, was incubated with 8 mL of 20 mM sodium citrate solution (pH 7.0), autoclaved at 121 °C and 1 atm for 30 min, and then centrifuged at 10,000× *g* for 15 min. By contrast, T-GRSP extraction was repeatedly extracted with 8 mL of 50 mM sodium citrate solution (pH 8.0) by autoclaving at 121 °C and 1.2 atm for 60 min, then centrifuging at 10,000× *g* for 15 min. The procedure of extraction was repeated four times until the extract appeared straw-colored, and all supernatants were collected. EE-GRSP and T-GRSP concentrations (mg per g of dry soil) were measured by the Bradford assay with the SP-2000 UV UV-Vis Spectrophotometer (Metertech Inc. Taipei. Taiwan) at 550 nm using bovine serum albumin as standard (Sigma-Aldrich Inc., St. Louis, MI, USA).

2.4. Chemical Determinations

The biomass samples were dried at 60 °C for 48 h, recording constant weight. The substrate samples were dried at 40 °C in an oven until constant weight, then ground and sieved to 2 mm. Subsequently, they were taken to 105 °C for 48 h to obtain a dry sample.

The concentrations of Cd, Cu, and Ni in plant biomass, substrate, and leached solution were analyzed by Inductively Coupled Plasma–Atomic Emission Spectroscopy (ICP-AES; HORIBA JOBIN YVON JY2000-2 model, Serial Number 0990/1236 02072618 NE); before ICP-AES analysis, dried root and shoot and substrate samples were digested with HNO_3/H_2O_2 and HCl according to Method 3050 B [21] and leached according to Method 3005 A in SW-846 [22].

For the determinations of phosphorus (P) in biomass, dried and ground plant samples were calcined at 500 °C. The ashes were dissolved in diluted HCl, and the P concentra-

tions were determined [23]. For P determination in soil, bicarbonate extraction was used following the Olsen method [24], with a UV 1100 UV–Vis Yoke spectrophotometer.

2.5. Phytomanagement Parameter Calculation

Bioaccumulation coefficients (BCs) and translocation factors (TFs) were calculated to determine the degree of metal(loid) accumulation in the shoots and roots of *B. salicifolia* with the following formulas:

$$\text{BC} = \text{metal(loid) concentration in plant shoot or root/metal(loid) concentration in final substrate,} \quad (6)$$

$$\text{TF} = \text{BC in shoot plant/BC in root plant} \quad (7)$$

BC > 1 indicates potential bioaccumulation, and TF > 1 indicates the translocation of metal(loid)s from root to shoot parts.

The bioextractive potential (BP) in the VDM was calculated as follows:

$$\text{BP (mg) BR} = \text{Concentration metal(loid) plant (ppm)} * \text{biomass (g) BR}/1000 \quad (8)$$

where biomass (g) BR is the mass of one plant * by the number of plants in the BRs, and the BP in the VDM is as follows:

$$\text{BP (g) VDM} = \text{BP (mg) BR}/10$$

$$\text{T-GRSP and EE-GRSP in the VDM were calculated with the following formulas:} \quad (9)$$

$$\text{T-GRSP or EE-GRSP (g)} = \text{concentration (g/g dry soil)} * \delta_s * V_{VDM}$$

where soil density (δ_s) is $\delta_s = 1.2924\ 10^6\ g/m^3$, and $V_{VDM} = 2.25\ m^3$.

2.6. Data Analysis

Data were subjected to an analysis of variance (ANOVA), and when significant differences among treatments were detected ($p < 0.05$), post hoc comparisons between mean values were made using Tukey's HSD test. Statistical procedures were performed with the Statistica 7.0 program.

3. Results

After four months in the BRs, most *B. salicifolia* plants in both treatments were colonized by AM fungi, either from propagules in the inoculum or from AM fungi already present in the BS, with typical AM structures developing within the roots (Figure 3). However, differences in mycorrhizal parameters were observed when inoculation was applied. The M+ plants exhibited a higher % F and % I of AM root colonization than M− plants in both the CS and BS treatments (Figure 4). However, M− plants were colonized by AM fungi present in the BS but at the lowest rate. Significant differences in % F were observed between inoculated and non-inoculated *B. salicifolia* plants, with no significant effects related to the soil substrate used in each treatment (Figure 4a). % F in M+ plants grown in CS (66.77 ± 15.35%) did not significantly differ from those grown in BS (69.16 ± 4.54%). In contrast, M− plants exhibited the lowest % F across both substrates with values of 23.7 ± 11.86% in CS and 5.53 ± 1.75% in BS, without significant statistical differences between substrates. The % I of AM root colonization in M+ plants grown in CS was significantly higher (55.45 ± 10.53%) compared to M− plants, both in CS (20.30 ± 10.53%) and BS (20.83 ± 5.58%) (Figure 4b). However, no significant differences in % I were observed between M+ plants grown in BS, which showed a value of 44.93 ± 8.94%.

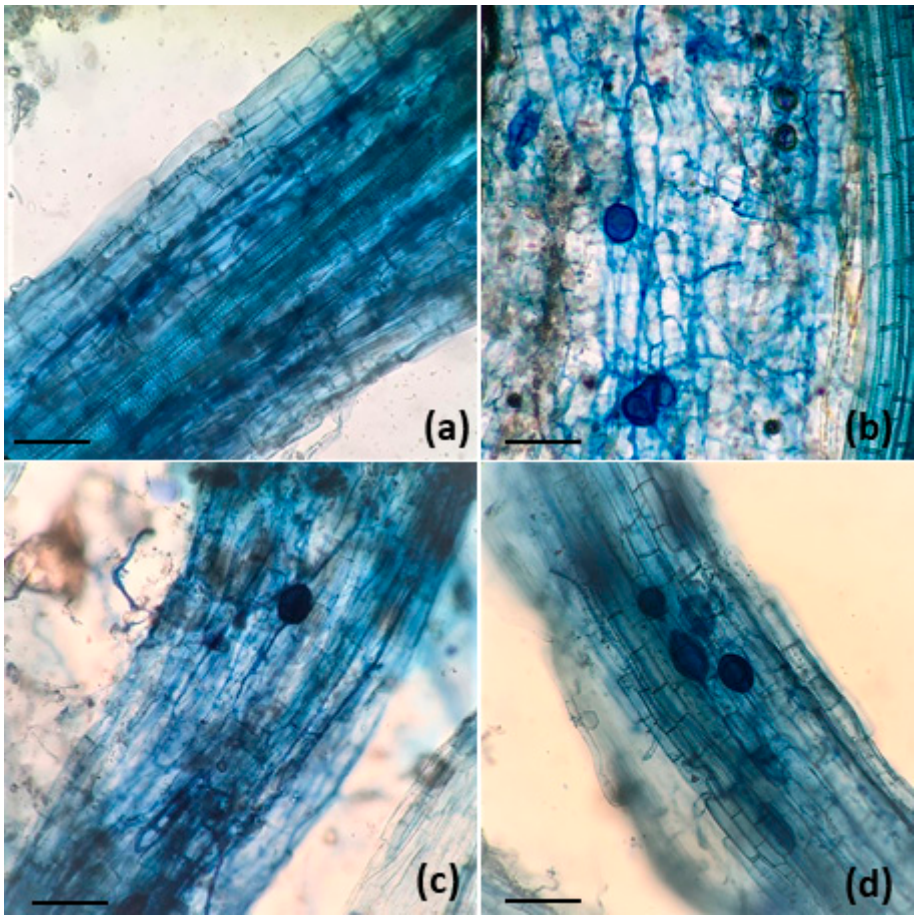

Figure 3. AM root colonization of *B. salicifolia* plants inoculated (M+) and non-inoculated (M−) developed in blank soil (BS) and contaminated soil (CS) in bioreactors. (**a**) Root fragment from BS M− treatment, bar = 100 µm. (**b**) Detail of spores and hyphae inside root from BS M+ treatment, bar = 80 µm. (**c**) AM root colonization by native AM species in CS M−, bar = 200 µm. (**d**) Detail of vesicles within root fragment from CS M+ treatment, bar = 200 µm.

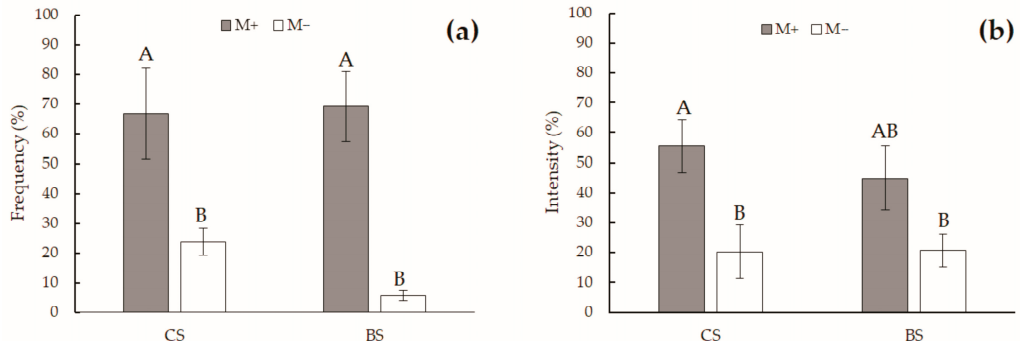

Figure 4. Percentage (%) of frequency (**a**) and intensity (**b**) of AM root colonization in *B. salicifolia* plants, inoculated (M+) and non-inoculated (M−) with mixture of AM species, when grown in contaminated soil (CS) and blank soil (BS) in bioreactors. Values are means ± standard error. Bars with different letters are significantly different (Tukey test, $p < 0.05$).

No significant differences were observed in the concentration of EE-GRSP estimated in the CS (2.46 ± 0.15 mg per g dry soil) and BS (2.22 ± 0.20) of M+ plants nor M− plants (Figure 5a). However, a higher content of EE-GRSP in the CS of M+ plants was registered than in the CS from M− plants. On the other hand, a higher concentration of EE-GRSP was quantified in the BS of M− plants (2.67 ± 0.38) compared to CS (1.49 ± 0.09). The

concentration of T-GRSP in the CS of M+ plants was significantly higher (1.94 ± 0.25 mg per g dry soil) than in M− plants in CS (0.46 ± 0.12) (Figure 5b). However, there was no statistically significant difference between the T-GRSP concentrations in the BS of M+ (1.21 ± 0.23) and M− plants (1.34 ± 0.35).

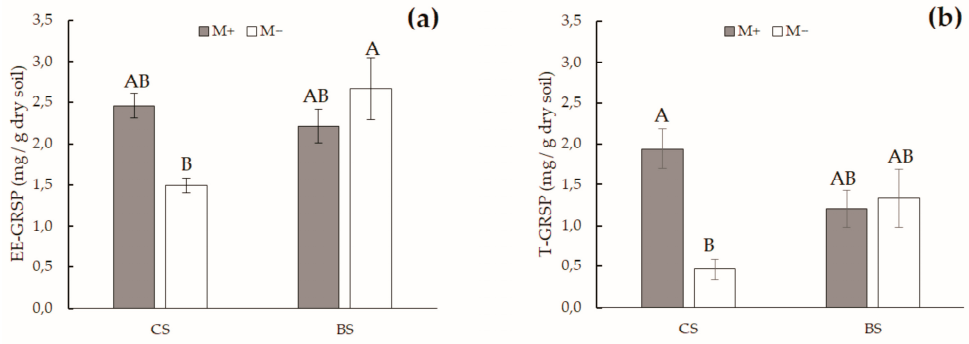

Figure 5. The concentration of easily extractable glomalin-related soil protein (EE-GRSP) (mg EE-GRSP per g dry soil) (**a**) and total glomalin-related soil protein (T-GRSP) (mg T-GRSP per g dry soil) (**b**) from the rhizosphere of *B. salicifolia* plants, inoculated (M+) and non-inoculated (M−) with a mixture of arbuscular mycorrhizal species when grown in contaminated soil (CS) and blank soil (BS) in the bioreactors. Values are the means ± standard error. Bars with different letters are significantly different (Tukey test, $p < 0.05$).

Table 2 shows the concentrations of Ni, Cd, Cu, and P in the shoot and roots of *B. salicifolia*, both inoculated with AM fungi and non-inoculated, growing in the BRs with BS or CS. The concentration in shoots was significantly higher in M+ plants grown in CS for Ni, Cd, Cu, and P (16.32, 153.69, 37.27, and 3358.52 ppm, respectively), while the same effect was found in Cu and P (10.45 and 2750.0 ppm, respectively) for the BS treatment. In roots, the concentration tends to be slightly higher in M− plants for Cd and Cu (13.55 and 6.84 ppm, respectively).

Table 2. The concentrations of Ni, Cd, Cu, and P in ppm (mean and between parentheses standard deviation) in contaminated (CS) and blank soil (BS) at the beginning and the end of the experiment, as well as in the biomass of *B. salicifolia* plants (root and shoot) and in the leached solution from the collector chambers.

Description	Ni	Cd	Cu	P
BS M+ shoot	BDL	BDL	10.45 ^{ab} (1.42)	2750.07 ^{c} (284.63)
BS M+ roots	BDL	BDL	16.08 ^{b} (1.60)	1717.71 ^{b} (200.15)
BS M− shoot	2.06 ^{a} (0.63)	BLD	1.09 ^{a} (0.15)	34.86 ^{a} (9.41)
BS M− roots	BDL	BLD	BDL	153.95 ^{a} (10.79)
CS M+ shoot	16.32 ^{b} (1.32)	153.69 ^{c}(16.71)	37.27 ^{c} (4.61)	3358.52 ^{c} (297.3)
CS M+ roots	BDL	5.21 ^{a}(0.3)	5.46 ^{a} (1.04)	103.91 ^{a} (13.72)
CS M− shoot	1.93 ^{a} (0,20)	31.14 ^{b} (2.5)	BDL	38.57 ^{a} (10.79)
CS M− roots	BDL	13.55 ^{ab} (1.9)	6.84 ^{ab}(1.43)	19.54 ^{a} (1.82)
BS initial	3.00 ^{c} (0.35)	BDL	11.75 ^{d} (1.75)	1000.58 ^{e} (100.42)
BS M+ final	3.13 ^{c}(0.81)	BDL	15.38 ^{d} (4.48)	1129.99 ^{e} (137.29)
BS M− final	3.00 ^{c} (1.04)	BDL	25.50 ^{d} (6.31)	1047.57 ^{e} (105.31)
CS initial	7.13 ^{d} (1.22)	2.62 ^{d} (0,09)	245.38 ^{g} (1.65)	585.07 ^{d} (107.20)
CS M+ final	6.50 ^{d} (1.04)	2.24 ^{e} (0.05)	175.25 ^{f}(5.81)	1123.82 ^{e} (83.10)
CS M− final	6.25 ^{d} (1.00)	2.08 ^{e} (0.03)	229.0 ^{g} (11.05)	1231.02 ^{e} (61.50)
Leached M−	0.26 ^{f} (0.02)	BDL	1.18 ^{h} (0.05)	1.27 ^{f} (0.01)
Leached M+	0.44 ^{g} (002)	BDL	1.39 ^{h} (0.08)	1.59 ^{g} (0.03)

ANOVA with post hoc Tukey HSD; in each column, different letters mean significant difference. BDL: Below Detection Limit; nd: not determined. CS: contaminated soil; BS: blank soil; M+: inoculated plants with AM fungi; M−: non-inoculated with AM fungi.

The concentrations of metal(loid)s leached into the collecting chamber at the end of the experiment were also recorded (Table 2). Ni and P concentrations significantly increased between the M+ and M− treatments (0.44 and 0.26 ppm for Ni; 1.59 and 1.27 ppm for P, respectively), whilst these amounts were negligible with the concentrations registered in plant biomass and soil. This indicated a stabilizing effect of the MAPm system for the elements under study given the low concentration detected in the leached solution from the collection chamber.

The shoot (aerial) and root bioaccumulation coefficients (ABC and RBC, respectively) and translocation factors (TFs) were calculated. Figure 6 shows the behavior of Cd, Ni, Cu, and P in plants with and without AM inoculation when grown in contaminated soil in the BRs. Bioaccumulation coefficients less than 1 were not considered, and those greater than 1 were highlighted. Higher ABCs in inoculated *B. salicifolia* were for Cd (68.61), followed by P (2.99) and Ni (2.51), while Cu was <1 (0.21), all of which were significantly higher than non-inoculated plants. On the other hand, a higher RBC for Cd in non-inoculated plants was found. In this sense, MAPm is highly effective for all the elements under study and is particularly promising for Cd and P.

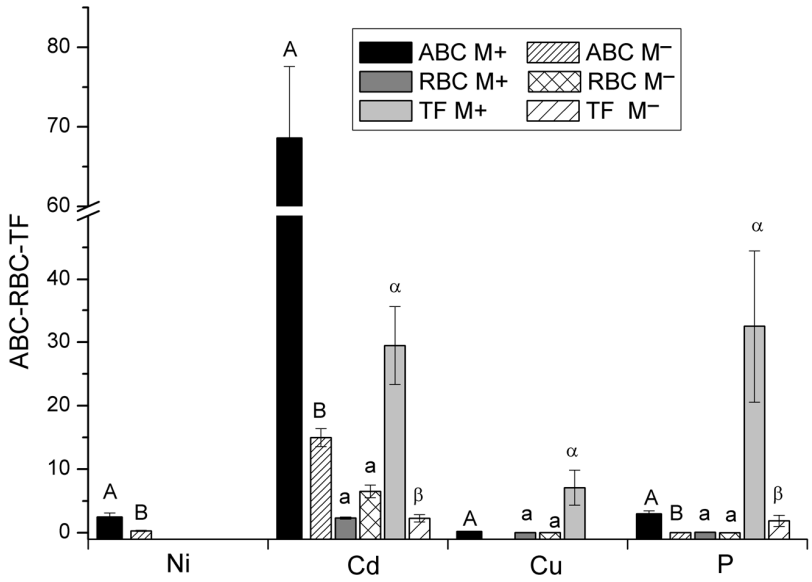

Figure 6. Shoot and root bioaccumulation coefficients and translocation factor (TF) for Ni, Cd, Cu, and P in *B. salicifolia* plants inoculated (M+) and non-inoculated (M−) with AM fungi in BRs. ABC: shoot bioaccumulation coefficient; RBC: root bioaccumulation coefficient. Values are the means ± standard error (vertical bars). For each element and parameter, bars with different letters are significantly different (Tukey test, $p < 0.05$).

The total plant biomass of the *B. salicifolia* inoculated output of BRs was 59.42 g per plant, and considering 12 plants in each BR, the total biomass estimated by each BR was 713.04 g (Table 3).

Table 3. The shoot and root biomass (g) of *B. salicifolia* plants grown in contaminated soil (CS) and blank soil (BS) with the inoculation (M+) and without the inoculation (M−) of AM fungi in the BR at the end of the experiment.

Treatment	Shoot Biomass (g)	Root Biomass (g)	Total Plant Biomass (g)
CS M+	52.67 (33.53)	6.75 (5.91)	59.42 (34.04)
CS M−	37.67 (28.31)	9 (7.21)	46.66 (29.21)
BS M+	30.00 (22.72)	5.50 (4.20)	35.50 (23.10)
BS M−	35.75 (24.06)	7.25 (2.87)	43.00 (24.23)

Values represent the mean and standard deviation between parentheses. CS: contaminated soil; BS: blank soil; M+: inoculated plants with AM fungi; M−: non-inoculated with AM fungi.

On the other hand, for the estimation of the bioextractive potential (BP) of the MAPm system in the BR and the VDM, 100 BRs involving one VDM concept are considered in Tables 3 and 4.

Table 4. The bioextractive potential (BP) of Ni, Cd, Cu, and P in the bioreactors (BRs) and the vegetable depuration module (VDM), when *B. salicifolia* was inoculated with AM fungi.

CRM and SRM	Concentration		Bioextractive Potential	
	Shoots *—Roots (ppm)	Mass/Plant (mg)	Total/BR (mg)	Total/VDM (g)
Ni	16.32 *	16.32	11.63	1.16
Cd	153.69 *—5.21	8.12	97.5	9.75
Cu	37.27 *—5.46	2.00	24.00	2.40
P	3358.52 *—103.9	177.6	2131	213.1

CRMs: Critical Raw Materials; SRMs: Strategical Raw Materials; * indicates concentration in shoots.

The projection of the results obtained in the BR for scaling up to the VDM is shown in Table 4. The mass of CRMs and SRMs that can be extracted in the VDM under the physicochemical and biological conditions of the experiment through four months of growth of inoculated *B. salicifolia* is indicated. BP was the highest for P (213.1 g), followed by Cd (9.75 g), Cu (2.40 g), and Ni (1.16 g). For scaling from the BR to the VDM, some variables were kept constant to achieve the same residence time of the water-soluble elements with the biomass and soil, to promote the same interaction in the BR and VDM of the phases: soil/biomass/water at both TRL levels. In this sense, the pH, Eh, porosity, and flow velocity (V_D and Va) in both the BR and VDM were approximately the same, while HWC, Q, and Tc were proportional. Table 5 shows the VDM experimental calibration values, based on measurements taken from those BRs.

Table 5. Hydraulic constants, physicochemical parameters, and the concentration of easily extractable glomalin-related soil proteins (EE-GRSPs) and total glomalin-related soil protein (T-GRSPs) estimated in the bioreactors (BRs) and the vegetable depuration module (VDM) under the CS M+ treatment.

Parameters	Qi (m^3/d)	Qe (m^3/d)	Q (m^3/d)	Ks (m^3/d/m^2)	Tc (seg)	V_D (m/d)	Va (m/d)	φ	WHC (kg)	pH	Eh	GRSP T (g)	EE (g)
BR	2.64	0.83	1.74	192.9	32	11.57	28.94	0.4	0.14	7.1	256	0.054	0.071
VDM	264	83	174	193	3200	11.57	28.94	0.4	14	7.1	225	5641	7150

Qi: income flow; Qe: egress flow; Q: average Qi and Qe; Ks: permeability constant; Tc: liquid retention time before exit to the collecting chamber; V_D: Darcy's velocity; Va: average linear velocity; φ: porosity; WHC: water holding capacity.

4. Discussion

The potential of AM fungi to promote plant growth, mitigate abiotic stress, and sequester metal(loid)s in plant biomass and/or soil is well known under laboratory or greenhouse conditions (TRL 2–3) but not fully harnessed at higher levels of scale, and as pointed out by Ibañez et al. [25], there are few trials of scaling to TRL 6. In the present work, we applied the MAPm system, consisting of *B. salicifolia* plants inoculated with a mixture of AM fungal species isolated from a Zn-Pb-Ag mine, into BRs (TRL 4) containing mine soil with high levels of Cd, Ni, and Cu, to decontaminate and recover CRMs and SRMs. This approach was then projected to the VDM at TRL 6 to analyze their effectiveness at higher levels.

Our MAPm system reached the highest bioaccumulation values of Cd, predominantly in the shoot biomass (ABC: 68.61) and roots (RBC: 2.33), with a significant translocation of this element towards the shoot biomass (TF: 29.45). Likewise, Cd accumulation in roots was significantly higher when plants were non-inoculated (13.55 ppm) compared with the inoculated one (5.21 ppm), indicating that inoculation increased translocation, resulting in high shoot and low root accumulation. Consequently, the MAPm system in the BRs

exhibited a phytoextraction behavior, taking up Cd from contaminated soil via AM hyphae, translocating it to the plant, and accumulating this element in the aerial plant biomass.

The effect of AM inoculation on the accumulation and translocation of heavy metal(loid)s was consistent with our previous studies, showing an increase in the TF values when *Helianthus annuus* was inoculated with the AM fungal species *Rhizophagus intraradices* (strain GA5) at different TRLs [17,18,26]. Salas-Luévano et al. [27] identified *B. salicifolia* developed under natural conditions as a potential tool for phytoremediation, although a lower Cd accumulation was reported (1.3 ppm of Cd in shoots and 6.6 ppm in soil). In contrast, Hard et al. [28] demonstrated that *B. salicifolia* from a polluted landfill is an effective bioaccumulator of Pb and Cd, which aligns with our findings. Previously, we reported high BCs and TFs of Zn (47.55 and 3, respectively) in *B. salicifolia* plants naturally developed in the CMFSR mine, thus demonstrating the great potential of *B. salicifolia plants* in bioremediation when they are studied at different TRLs [13].

The ability to translocate Cd to the shoot biomass and excrete it through leaf trichomes was proposed as a tolerance mechanism of metal stress by plants [29]. However, the role of AM fungal species involved in assisting phytoremediation is critical. In previous work, we observed a stabilizing effect, with decreased TF values in inoculated *Senecio bonariensis* (*Asteraceae* family) plants when different AM fungal strains were used in the VDM, thus demonstrating the importance of the appropriate selection of AM fungi in bioremediation process [18]. The AM fungal species used to inoculate *B. salicifolia* plants, isolated from a polluted mine (*Paramillos de Uspallata*), revealed a greater tolerance by its intense root colonization compared to the AM species already present in the soil. Several studies have shown that AM fungi isolated from metal-contaminated soils often exhibit greater tolerance to pollutants than species from less contaminated environments [30]. Therefore, the AM species from the *Paramillos* mine are valuable biological resources for phytoremediation applications. In this study, the BP of metal(loid)s estimated for the MAPm system in the VDM was 1.16 g Ni < 2.40 g Cu, <9,75 g Cd < 213.1 g P, greater values than those obtained in previous works by using other plant species and AM fungi, registering 0.216 g Ni < 0.5 g Cu < 114 g P [26]. To optimize Cd removal efficiency in the MAPm system, co-inoculation with other beneficial microorganisms should be applied in future studies [31].

AM fungi typically enhance plant growth in Cd-contaminated soils by mobilizing available P from soil through their external mycelial networks, thus making them useful for remediation [32]. Our results showed a significantly higher uptake and translocation of P in inoculated plants (ABC M+: 2.99) than in non-inoculated plants (ABC M−: 0.125), in both contaminated and blank soil. In addition, mycorrhizal plants enhance the production of molecules that chelate Cd by forming complexes, including phytochelatins, metallothioneins, and glutathione [3,12,29,32]. Glomalin produced by AM fungal mycelia can also bind more metals, significantly immobilizing them and promoting host plant tolerance to harsh situations. We found that the concentrations of EE-GRSP and T-GRSP were higher in inoculated plants than in non-inoculated plants grown in contaminated soil. Increments in glomalin, and the high frequency and colonization intensity rates, may be attributed to the AM species used in inoculation, and a positive correlation between EE-GRSP and AMF biomass may result in great glomalin exudation [15].

When *B. salicifolia* was inoculated, Ni concentration significantly increased in aboveground biomass (16.32 g M+ and 1.93 M−). This was also observed in Cu accumulation and translocation, while soil concentration decreased significantly in Cu and Cd. Both Ni and Cu are classified as SRMs included in the fifth list of CRMs, along with P, making their recovery from biomass through hydrometallurgical techniques, by the purification with selective electrodeposition, thus permitting a sustainable and selective metal recovery at a high degree of purity (99%) and determining commercial reuse. By applying a leaching/purification processing circuit of the SRMs and CRMs accumulated and concentrated on plant biomass, a recovery of 90% of purified metals was demonstrated [33]. In this sense, CRMs from biomass (1.16 g of Ni, 2.40 g of Cu, and 213.1 g of P) could be recovered through the VDM. Element recovery, in gram order, was also recorded in Scotti et al. [17],

where 12.4 g of Mn, 29.3 g of Zn, 17.6 g of Sr, and 5 g of Cu in the biomass of the VDM were extracted. Contrarily, these elements were phytostabilized and were not efficiently extracted depending on the AM fungal strain used in Colombo et al. [18]. This may be due to various mechanisms involved in the entry of elements into the microorganisms and roots, such as the bioavailability of elements in the soil. One of the main mechanisms that limit bioavailability is the accumulation of heavy metal(loid)s in the spores and mycelium of AM fungi. These effects might interfere with the decrease in the concentration of the elements in the soil but could also impact their leaching. In our work, we found a greater output of P and Ni to the collection chamber when inoculation occurred, and only the concentration of Cd in the soil decreased. This result could be related to the microbial activity that could increase in the mycorrhizosphere because of the abundance changes in microorganisms associated with AM hyphae, such as P solubilizers and Ni solubilizer bacteria [34,35].

The calibration of the physical, chemical, and biological parameters was important for scaling the MAPm system from TRL 4 to TRL 6. In this sense, Ibañez et al. [25] discussed the importance of the tests at TRL 6, and Xu et al. [36] used the retention time in BRs simulating artificial wetlands with a water inlet in vertical flow to analyze metal uptake effects in mycorrhized plants. They also discussed the importance of the BR filling system, permeability, and porosity. In this sense, it is important to keep the chosen parameters within the conditions required by the applied law. We considered laminar vertical flow with aeration at the time of the water inlet, applying Darcy's law in the VDM as outlined previously [17].

The behavior of a constructed wetland mainly depends on the hydraulics, chemistry, and permeability of the substrate [37]. A common obstacle encountered in constructed wetlands is an inadequate oxygen supply, which causes stressful conditions for plants and microorganisms and decreases metal removal efficiency [38,39]. The characteristics of the proposed VDM that favor an aerobic environment and remove CRMs and SRMs include vertical subsurface flow [39], appropriate potential redox and pH values [40], a high density of root biomass, and the development of an extraradical mycelial network in the substrate [41]. We accounted for the main variables necessary for scaling and considerations related to Darcy's law.

5. Conclusions

The proposed MAPm system demonstrated a significant strategy for effectively extracting Cd from soils while maintaining P concentration, making it a promising option for intercropping in contaminated soils. Additionally, MAPm enhances the translocation of the studied elements, positioning it as a viable strategy for CRM and SRM recovery through hydrometallurgical processes.

The calibration parameters used allowed for the scaling up of MAPm into the BRs under similar hydraulic, chemical, and biological conditions.

This methodology can be tested with other elements according to industrial requirements. In this sense, we are making adjustments to bring this biotechnology to the territory to solve the Cd and cocoa problem in Ecuador, as well as the Ni, Cu, and U problems in the Pilcaniyeu Technological Complex (Argentina) for the restoration of natural wetlands. This biotechnology is very economical. The biomass waste is processed by hydrometallurgy, obtaining a recovery of 90% of pure metals (99% purity), which is important when calculating costs and benefits. The patent that was used covers many chemical elements, but the challenge is in achieving the physical/chemical/biological conditions that promote the desired behavior of the chemical elements.

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Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Glossary of bio-terms and physical terms:

Colonized roots: This refers to the establishment and spread of arbuscular mycorrhizal fungi within plant roots.
Sporulation: This refers to the formation of spores, asexual reproductive and dispersal units, that originate from a hypha of arbuscular mycorrhizal fungi.
Permeability hydraulic constant (K): This refers to the hydraulic conductivity or permeability of the soil or substrate. It is a symmetrical tensor that can be diagonalized into three main directions. The water flow will move along in the direction of greatest permeability, and this in turn will indicate the speed value at which water moves under a unit gradient condition.
Porosity: This refers to the percentage of void space within a material. For example, the percentage of empty space within a box of soil or the percentage of empty space within a given rock is known as porosity.
Percentage of frequency and intensity: The frequency of arbuscular mycorrhizal colonization in roots is determined as the number of root segments containing any intraradical AM structure (hyphae, arbuscules, coils, or vesicles) over the total root segments, and the intensity is defined as the extensive rate of colonization in a root segment.
Easily extractable glomalin-related soil protein: This refers to the newly produced glomalin fraction, a glycoprotein produced by arbuscular mycorrhizal fungi, that is extracted from soil by autoclaving once in 20 mM sodium citrate buffer (pH 7.0).
Total glomalin-related soil protein: The recalcitrant soil glomalin fraction, a glycoprotein produced by arbuscular mycorrhizal fungi, extracted by autoclaving soil repeatedly in 50 mM sodium citrate buffer (pH 8.0), until the extract appears straw-colored.

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