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Microbial inoculants alleviate the adverse effects of Cu-contaminated soils amended with biochar on sunflower growth

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A B S T R A C T

Soil contamination is a pressing global issue driven by various anthropogenic activities, such as mining. This study evaluated the use of biochar and microbial inoculants as phytoremediation allies in promoting sunflower growth in a Cu-contaminated mining soil. Sunflower seedlings were planted in a Cu-contaminated mining soil amended with increasing doses of biochar (0 %, 2.5 %, and 5 % w/w) under greenhouse conditions. Seedlings were singly and co-inoculated with the bacterial strain *Pseudomonas reactans* EDP28 and the commercial arbuscular mycorrhizal fungi (AMF) *Rhizophagus irregularis*.

The addition of 2.5 and 5 % of biochar to the Cu-contaminated mining soil significantly reduced sunflower shoot biomass by 49 % and 46 %, respectively, and root biomass by 63 and 50 %, respectively. This decrease is likely attributed to increased Cu accumulation in plant tissues, particularly in the roots (on average +38 %), driven by the enhanced availability of Cu in the soil. However, microbial inoculation, particularly the combined application of the bacterial strain and the AMF (Mix treatment), significantly supported sunflower growth and resilience under metal stress conditions. Mix treatment improved root elongation, root biomass, and shoot biomass by 48 %, 143 %, and 122 % at 2.5 % biochar, and by 45 %, 54 %, and 137 % at 5 % biochar, respectively. This was achieved by improving chlorophyll content and nutrient use efficiency. The beneficial effects were clearer in soils without biochar addition, where inoculation fully promoted sunflower growth. In contrast, in biochar-amended soils, inoculation helped to partially counteract the negative effects of biochar on plant development.

This study demonstrates that sunflowers can effectively tolerate and accumulate high levels of Cu in their tissues, making them a promising candidate for phytoremediation strategies in mining areas, especially when aided by microbial inoculants, whilst the role of biochar in phytoremediation requires further investigation. Biochar can facilitate metal accumulation, but its impact on plant growth needs careful management. Future research should focus on optimizing the application rates and combinations of biochar and microbial inoculants to maximize phytoremediation efficiency and minimize any adverse effects on plant growth.

1. Introduction

Soil contamination significantly hinders the achievement of United Nations Sustainable Development Goals, threatening human health and ecosystem sustainability (FAO and UNEP, 2021; Liu et al., 2023). Organic and inorganic pollutants, particularly from mining industry (Li et al., 2019a; Masindi and Muedi, 2018), impair soil functions and ecosystem services (González-Morales et al., 2022). Open-air tailings ponds in the vicinity of mines (Mendez and Maier, 2008; Wong, 2003) expose residues to atmospheric conditions, dispersing pollutants, including metals, into surrounding areas (Benidire et al., 2021; Khalil et al., 2008; Mokhtari et al., 2018). This issue demands urgent attention as even low metal concentrations can cause environmental disturbances and health risks through biomagnification (Li et al., 2019a). In response, soil contamination has become a focus of European Union policies, including the European Green Deal. Initiatives like the Farm to Fork strategy aim to reduce chemical pesticides and fertilizers by 50 % and 20 %, respectively, by 2030, while the Biodiversity Strategy emphasizes restoring contaminated sites and mitigating land degradation (Montanarella and Panagos, 2021).

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Several conventional remediation techniques have been employed in metal-contaminated sites, including excavation followed by disposal in controlled landfills, incineration, chemical washing, vitrification, among others (Azhar et al., 2022; Li et al., 2019a; Liu et al., 2018; Wang et al., 2017). However, these methods have drawbacks such as high implementation costs and the degradation of soil functions (Burges et al., 2018; Lacalle et al., 2020). In response to these challenges, plant-based remediation options, i.e. phytoremediation, have gained ground in recent decades. Phytoremediation is acknowledged as a green alternative to conventional techniques because it is cost-effective, scalable for large-scale implementation (Liu et al., 2018), while causing minimal soil disturbance, which helps preserve soil functions and ecosystem services (Lacalle et al., 2020).

Energy crops can contribute significantly to the economic and environmental value of metal-contaminated soils by serving as a solution for both biomass production and soil remediation (Burges et al., 2018; Moreira et al., 2021). Among these, sunflower (*Helianthus annuus* L.) emerges as a particularly promising candidate. It is an oilseed crop with high economic value, and its biomass can be used as raw material for various bioenergy purposes (Moreira et al., 2021; Zhou et al., 2020).

In addition to contamination, mining soils often present additional challenges such as low levels of nutrients and organic matter, poor structure, low pH, and/or high salinity (Alvarenga et al., 2022; Benidire et al., 2020). To tackle these issues and facilitate the successful establishment of plants in mining soils, various strategies can be employed, including the application of organic amendments and microbial inoculants. Organic amendments are designed to immobilize metals and enhance physicochemical and nutritional soil properties, thereby creating optimal conditions for plant growth (Ferreira et al., 2022; Gao et al., 2020). Among these, biochar has gained widespread use as an amendment in metal-contaminated soils, due its ability to adsorb metals, thereby reducing its bioavailability and mobility in soil (Beesley et al., 2014; Fan et al., 2023; Li et al., 2019b; Wang et al., 2019). However, the efficiency of biochar in remediation depends on various factors such as pyrolysis temperature, the composition of the raw material used in its production, and the types of metals and soil properties involved (Fan et al., 2023; Xu et al., 2020). For instance, biochar derived from peanut shells, straw blocks, and maize straw has demonstrated greater effectiveness in reducing the exchangeable forms of Cd and Zn compared to biochar derived from pine and wood pellets (Xu et al., 2020). Furthermore, the effectiveness of biochar in immobilizing metals tends to increase with decreasing particle size (Fahmi et al., 2018).

Microbial inoculants, such as plant growth-promoting rhizobacteria (PGPR), directly enhance plant growth and yield by promoting nutrient uptake (e.g., N_2 fixation, P and K solubilization), increasing phytohormone levels (e.g., indole-acetic acid (IAA)), and reducing stress ethylene levels. Recent studies have demonstrated the beneficial effects of microbial inoculants on the growth and resilience of energy crops cultivated in metal-contaminated areas (Chen et al., 2022; Iqbal et al., 2023; Ke et al., 2021; Montreemuk et al., 2024). Furthermore, the complementary mechanisms of both biochar and microbial inoculants may leverage the effectiveness of phytoremediation strategies by improving plant growth, increasing metal immobilization, and enhancing soil health (Haider et al., 2022; Ouyang et al., 2023; Tu et al., 2020; Wang et al., 2017). Despite these positive effects, there is a notable lack of studies investigating synergetic interactions between soil amendments such as biochar and PGPB and AMF under diverse soil conditions, plant species, and contamination types. These combinations have the potential to significantly influence metal uptake or immobilization, plant resilience to metal stress, and optimize biomass production, factors that are essential for the success and scalability of phytoremediation strategies.

This study aimed to explore the effect of single and dual inoculation with the PGPR *Pseudomonas reactans* EDP28 and the AMF *Rhizoglomus irregulare*, along with different application rates (2.5 and 5 %) of biochar, on the growth of sunflower in a mining Cu-contaminated soil. Specifically, the study investigated how these treatments influenced sunflower biomass production and Cu and nutrient uptake in the presence of contamination. The selection of beneficial soil microorganisms was based on prior studies. The bacterial strain EDP28, isolated from metal-contaminated soil in northern Portugal, demonstrated high resistance to Cd and Zn (Pires et al., 2017) and exhibits key plant growth-promoting (PGP) traits, such as high ACC-deaminase activity and siderophore production (Pereira et al., 2015). Moreover, it has shown to effectively enhance *in vitro* growth of maize seedlings exposed to increasing concentrations of Zn and Cd. These bacterial strains also promoted the accumulation of both metals and improved the nutritional status of maize plants grown in a multi-contaminated mining soil (Moreira et al., 2016a). Similarly, the AMF *R. irregulare* acted as plant-promoting inoculant, enhancing shoot and root biomass, as well as shoot elongation of maize plants grown in a metal contaminated soil (Moreira et al., 2016b).

2. Material and methods

2.1. Soil sampling and analysis

A composite soil sample consisting of 5 sub samples from the top layer (0–20 cm) was randomly collected from an experimental field established under the scope of the Phy2SUDOE project at the Borralha mine, Salto, Montalegre, Portugal. Intensive mining activities have led to the generation of tailings containing high concentrations of potentially hazardous trace elements, such as Cu (Ávila et al., 2015). These tailings have been exposed to local atmospheric conditions, resulting in the dispersion of contaminants into the surrounding areas (Ávila et al., 2015).

The soil was air dried, thoroughly mixed, and sieved (<2 mm) for physicochemical characterization. Soil properties were as follows: pH 4.45 ± 0.01 (potentiometric); electric conductivity 150 ± 2 (μS cm^{-1}; conductimetry); organic matter content 7.12 ± 0.01 (%; Walkley-Black); organic carbon 4.13 ± 0.04 (%; conductimetry), texture silt loam (hydrometer); total N 0.31 ± 0.02 (%; catarometry); CEC 2.80 ± 0.10 ($cmol^{+}kg^{-1}$; hexamminecobalt chloride); extractable K 129.60 ± 1.30 (mg kg^{-1}; mehlich 3); extractable Mg 64.20 ± 0.40 (mg kg^{-1}; mehlich 3); extractable Ca 540.9 ± 0.9 (mg kg^{-1}; mehlich 3); extractable P 437.00 ± 0.40 (mg kg^{-1}; mehlich 3); pseudo-total Cd 1.45 ± 0.05 (mg kg^{-1}; aqua regia); pseudo-total Cu 1080.00 ± 0.50 (mg kg^{-1}; aqua regia); pseudo-total Pb 71.06 ± 0.05 (mg kg^{-1}; aqua regia); pseudo-total Zn 228.24 ± 0.70 (mg kg^{-1}; aqua regia).

2.2. Microbial inoculants

The rhizobacteria *Pseudomonas reactans* EDP28 and the commercial AMF *R. irregulare* obtained from the company INOQ (Germany), were used as microbial inoculants. The bacterial strain EDP28 was grown in tryptic soy broth (TSB) medium at 30 °C with agitation at 100 rpm until it reached the exponential growth phase. The inoculum density was c.a. 10^8 CFU ml^{-1}. The AMF *R. irregulare,* containing 145 mycorrhizal units cm^{-3} of vermiculite (1–2 mm), was mixed in the soil according to the recommendations of manufacture (100 ml kg^{-1} soil) five days before seedling transference.

2.3. Pot experimental design

The pot experiment was conducted in a controlled growth room (12 h photoperiod, 450 μmol m^{-2} s^{-1} photosynthetically active radiation, 18–21 °C temperature range, 50–60 % relative humidity range) at Centro de Biotecnologia e Química Fina, Universidade Católica Portuguesa, Porto, Portugal for 12 weeks, corresponding to the onset of sunflower flowering. The experiment consisted of a factorial design with four microbial treatments: C – control - non-inoculated; B – rhizobacteria *P. reactans*; F – AMF *R. irregulare*; Mix – mixture of *P. reactans* and

R. irregulare and three doses of biochar: 0, 2.5, and 5 (w/w) with five replicates each.

Biochar (Ecochar®) was purchased to the company Ibero Massa Florestal, S.A., Portugal (Ecochar® Technical Sheet 2024) and had the following characteristics: particle size 1–10 mm, pH 8–10, total fixed C $\geq$ 90 %, total N $\leq$ 5 g kg^{-1}, total Cd < 0.05 mg kg^{-1}; total Pb 0.05 mg kg^{-1}; total Fe 99.5 mg kg^{-1}, total As and Hg < 0.01 mg kg^{-1}, ashes $\leq$ 5 %, humidity < 30 %, volatiles < 5 %, (Martins et al., 2022; Arrobas et al., 2021). This biochar was produced in a pyrolytic reactor using wood biomass derived from silver wattle (*Acacia dealbata*) (Arrobas et al., 2021).

Sunflower seeds cv. Bosfora, purchased to Syngenta Spain, S.A, were surface sterilized using a solution of NaOCl (v/v) for 10 min and then rinsed with deionized sterilized water (at least 6 rinses). Seeds were then sowed in water-agar for 7 days. Six sunflower seedlings were transferred to plastic pots containing 1 kg of soil or soil amended with biochar. Seedlings were thinned to five after one week. One week after transference of seedlings to pots, 25 ml of bacterial suspension (ca. 10^8 CFU ml^{-1}) were sprayed onto the soil surface (B and Mix treatments), while 25 ml of a diluted and non-inoculated TSB solution (1:1 sterilized water/TSB) were applied in pots corresponding to C and F treatments. A re-inoculation was conducted four weeks after seedling transplantation as previously described. Pots were irrigated three times a week with tap water.

2.4. Plant analysis – chlorophyll content, biomass production, Cu and nutrient accumulation

A Soil Plant Analysis Development (SPAD) chlorophyll meter (Spectrum Technologies, Inc) was used to estimate the chlorophyll content of leaves at harvest (12 weeks). Three readings were taken on the second fully expanded leaf of each plant.

At harvest, shoot elongation was measured and plants were separated in roots and shoots. Roots were then carefully washed with tap water, followed by a solution of 0.1 M HCl, and finally with deionized water to remove soil and/or biochar particles.

Plant tissues were oven dried at 60 °C for 2 weeks to determine shoot and root dry biomass. Samples were ground (Culatti, Micro Impact Mill) and digested (Berghof Speed Wave MWS-3 + Microwave Digestion System) using a H_2SO_4:H_2O_2 (1:1) mixture.

The concentrations of Cu in plant tissues (roots and shoots) were determined by Flame Atomic Absorption Spectroscopy (FAAS) using a Unicam-969 AA Spectrometer (Waltham, USA). A biological standard reference material (1573a tomato leaves) provided by the National Institute of Standards and Technology was used to confirm the accuracy and precision of methods by comparison with certified values of each element. Root and shoot bioconcentration factor (BCF) and translocation factors (TF) were determined for Cu as described by Ali et al., (2013).

The total P and N contents in roots and shoots were determined by colorimetry following the method of Walinga et al. (1989). For N determination, two reagents were sequentially added to the digested sample: 3 ml of reagent 1, containing a mixture of 50 mM disodium hydrogen phosphate buffer (pH 12.3) and 4 % bleach solution, followed by 5 ml of reagent 2, comprising 1 M sodium salicylate, 1 mM sodium nitroprusside, and 3 mM EDTA. For total P determination, two reagents were added to the digested samples: 3 ml of reagent 1 (30 mM ascorbic acid solution) and 5 ml of reagent 2 comprising a mixture of 6 mM antimonyl tartarate, 5 mM ammonium molybdate and 0.7 M sulphuric acid solutions, and an anticoagulant agent (Wetting aerosol 22, USA). N and P concentrations were determined at 660 nm and 880 nm, respectively, using a UNICAM HELIOS spectrophotometer (Waltham, USA).

The physiological N (NUE) and P (PUE) use efficiency were determined according to the formula (Nguyen et al., 2014): Nutrient use efficiency = Total dry biomass / Total nutrient absorbed, where Total nutrient absorbed = Nutrient concentration x Total dry biomass.

2.5. Cu bioavailability in soil

Rhizospheric soil samples were collected from each pot at harvest for the determination of Cu extractable forms. According to De Koe (1994), Milli-Q water and ammonium acetate (NH_4- Ac) - extractable metal fractions were obtained mixing 2.5 g of soil with 12.5 ml of water and 1 M NH_4 –Ac, respectively. The suspensions were shaken at 150 rpm and incubated at 30 °C for 1 h, after which they were centrifuged and filtered through a 0.45 μm cellulose acetate filter (Sartorius). Cu extractable forms were determined by FAAS.

2.6. Data analysis

Statistical analyses were performed using the statistical software package SPSS 26.0 (SPSS Inc., Chicago, IL, USA) and R (version 4.3.3). Two-way ANOVA was performed to assess the significant differences of the effect of microbial treatments and biochar doses on each parameter. One-way ANOVA with Duncan post hoc analysis was also performed to assess the effects of microbial inoculants on the different plant parameters for each dose of biochar. Pearson correlation matrix and the significance levels were performed using the R function *rcorr* () from the package *Hmisc*. A correlogram with significance levels was created by using the function *corrplot* () from the *corrplot* package.

3. Results

3.1. Plant parameters – chlorophyll content, shoot elongation and biomass production

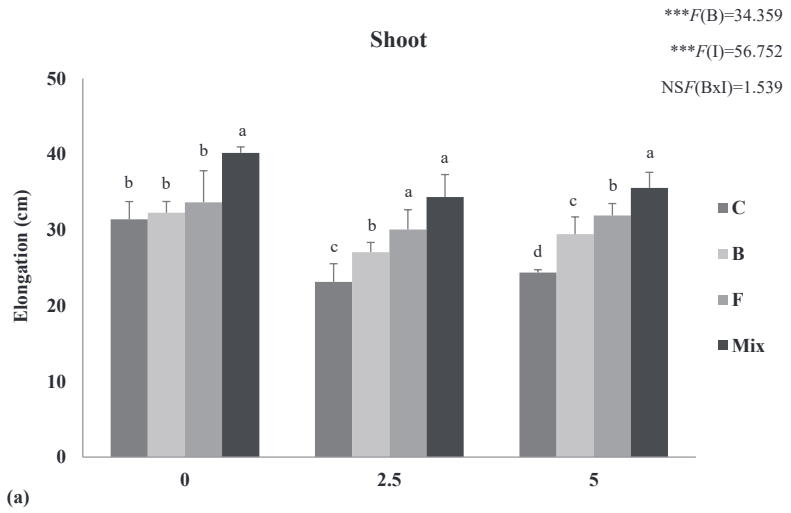
SPAD readings revealed variations in chlorophyll content between non-inoculated and inoculated plants cultivated in biochar-amended soils, ranging from 30.34 to 36.14 (Table 1). The addition of biochar had minimal impact on chlorophyll content, whereas microbial inoculants tended to enhance it, with the greatest effect observed in plants co-inoculated with AMF and the rhizobacteria EDP28 (Mix).

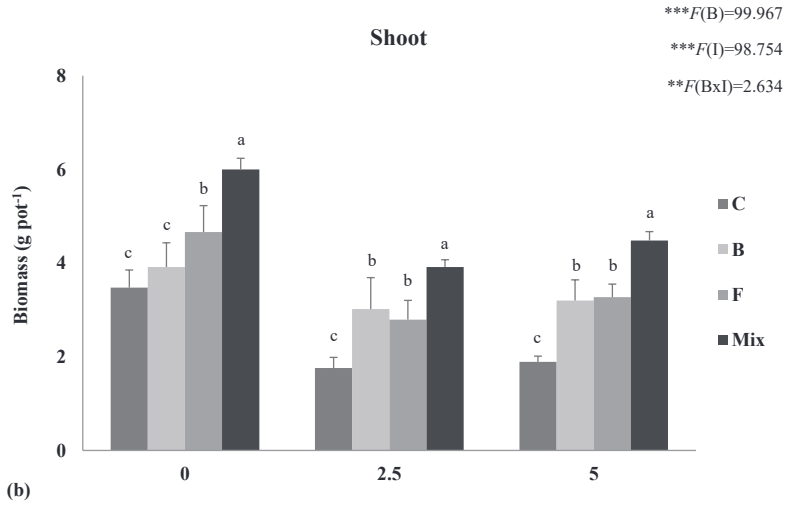
The effect of biochar and microbial inoculants on sunflower's growth is presented in Fig. 1. Shoot elongation varied between 23.16 and 40.20 cm (Fig. 1a). The addition of 2.5 % and 5 % of biochar to mining soil resulted in an average reduction in shoot elongation of 26 % and 22 %, respectively, compared to plants grown in non-amended soil.

Table 1
SPAD readings of leaves of sunflower plants grown in a mining soil amended with different biochar doses (0, 2.5, and 5 %) and treated with different microbial inoculants: (C – control – non-inoculated; B – rhizobacteria *P. reactans;* F – AMF *R. irregulare*; Mix – mixture of *P. reactans* and *R. irregulare*) at the end of the experiment (harvest).

	Biochar (%)		
Inocula	**0**	**2.5**	**5**
C	31.78 ± 1.09^{c}	30.34 ± 1.24^{b}	31.91 ± 0.79^{c}
B	34.30 ± 1.07^{b}	31.31 ± 1.67^{b}	33.37 ± 0.95^{a,b}
F	34.96 ± 0.35^{a,b}	31.50 ± 0.93^{b}	32.20 ± 0.82^{b,c}
Mix	36.14 ± 1.45^{a}	33.94 ± 1.60^{a}	34.39 ± 1.16^{a}
	* **F= 14.883	* *F= 6.055	* *F= 7.319
* **F(B)= 24.118			
* **F(I)= 23.023			
NSF(BxI)= 1.797			

Values are means ± standard deviation (n = 5). A two-way ANOVA was performed to determine the influence of biochar and of microbial inoculants on chlorophyll content. The results are shown with the test statistic for each case (B: biochar; I: microbial inoculants; B x I: biochar x microbial inoculants) and as NS: Non significant at the level P > 0.05; * significant at the level P < 0.05; * * P < 0.01; * ** significant at the level P < 0.001, respectively. One-way ANOVA was performed to determine the influence of microbial inoculants on SPAD readings for each biochar dose. Means for the same dose showing different letters are significantly different from each other (P < 0.05) according to Duncan test.





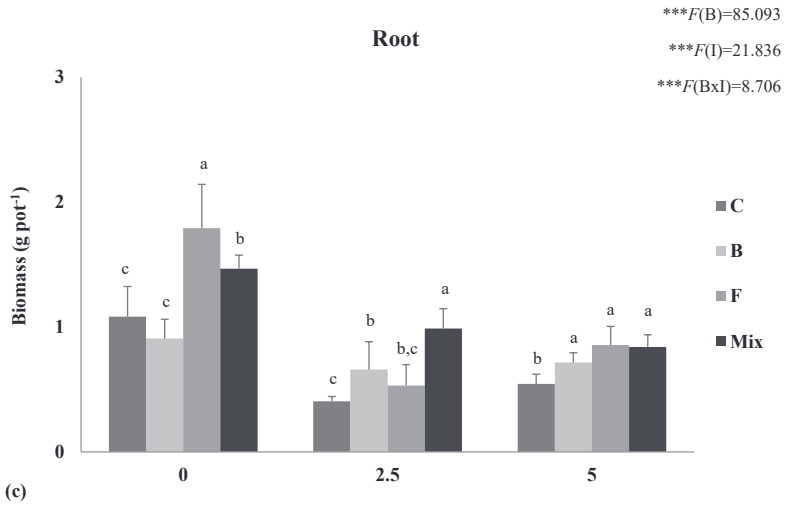


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Fig. 1. Shoot elongation (a), shoot (b) and root (c) dry biomass (mg kg^{-1}) of sunflower plants grown in a mining soil amended with different biochar doses (0, 2.5, and 5 %) and treated with different microbial inoculants: (C – control – non-inoculated; B – rhizobacteria *P. reactans;* F – AMF *R. irregulare*; Mix – mixture of *P. reactans* and *R. irregulare*). Values are means ± standard deviation (n = 5). A two-way ANOVA was performed to determine the influence of biochar and of microbial inoculants on shoot elongation and shoot and root dry biomass of sunflower plants. The results are shown with the test statistic for each case (B: biochar; I: microbial inoculants; B x I: biochar x microbial inoculants) and as NS: Non-significant at the level $P > 0.05$; * significant at the level $P < 0.05$; * * $P < 0.01$; * ** significant at the level $P < 0.001$, respectively. One-way ANOVA was performed to determine the influence of microbial inoculants on shoot elongation and shoot and root dry biomass for each biochar dose. Means for the same dose showing different letters are significantly different ($P < 0.05$) from each other according to Duncan test. For elongation, the F values of one-way ANOVA are * **F= 12.389, * **F= 19.619, * **F= 36.554, respectively for 0, 2.5, and 5 % of biochar. For dry shoot biomass, the F values of one-way ANOVA are * **F= 31.148, * **F= 22.658, * **F= 68.829, respectively for 0, 2.5, and 5 % of biochar. For dry root biomass, the F values of one-way ANOVA are * **F= 14.365, * **F= 11.957, * **F= 9.402, respectively for 0, 2.5, and 5 % of biochar.

However, the application of microbial inoculants, particularly when applied as a consortium (Mix treatment), counteracted this decline. Shoot dry biomass ranged from 1.76 to 5.99 g (Fig. 1b) while root dry biomass varied between 0.41 and 1.79 g (Fig. 1c). Plants grown in soil enriched with 2.5 and 5 % of biochar experienced reductions of 49 % and 46 % respectively, compared to plants cultivated in biochar-free soil. A similar trend was observed for root growth (Fig. 1c), with the addition of 2.5 % and 5 % of biochar resulting in decreases in biomass production by 63 % and 50 % respectively. Microbial inoculation, particularly the combined application of AMF and PGPB, mitigated these declines in both plant organs. However, despite the beneficial impacts of microbial treatments on shoot and root biomass, inoculation did not fully counteract the growth reduction observed at 2.5 % and 5 % of biochar.

3.2. Cu accumulation in plant tissues

The accumulation of Cu in roots and shoots of sunflower is illustrated in Fig. 2. Cu concentrations in roots ranged from 406.48 to 912.98 mg kg^{-1} (Fig. 2b), which was approximately 12 times higher than concentrations in the shoots, ranging from 38.05 to 60.36 mg kg^{-1}). These findings are supported by the low TF observed across all treatments (Table 2). Non-inoculated plants cultivated in soils enriched with 2.5 and 5 % of biochar exhibited a significant increase in Cu accumulation in roots, averaging 38 % more than non-inoculated plants grown in non-amended soil. At 2.5 % of biochar, both single and mixed AMF inoculation increased Cu concentration in roots by 21 and 51 %, respectively. Consistently with the Cu accumulation pattern, the BCF of Cu was higher in sunflower roots compared to shoots (Table 2). Moreover, increasing doses of biochar significantly decreased TF of Cu.

3.3. Plant nutritional status

The impact of biochar and microbial inoculants on NUE and PUE is shown in Table 3. On average, NUE increased by 10 and 20 % in plants cultivated in soil amended with 2.5 and 5 % of biochar, respectively, compared to those grown in non-amended soil. NUE was further enhanced in plants inoculated with a mixture of PGPB and AMF (Mix treatment) in non-amended soil. The addition of biochar also improved PUE. Overall, inoculated plants showed enhanced PUE, with the most significant improvement observed in the Mix treatment.

3.4. Extractable Cu concentration in soil

The NH_4-Ac-extractable soil fraction of Cu is shown in Table 2, with concentrations ranging from 10.88 to 21.53 mg kg^{-1}. The addition of 2.5 % and 5 % of biochar significantly increased Cu availability by 58 % and 76 %, respectively. In contrast, overall, inoculation marginally decreased Cu availability in the soils.

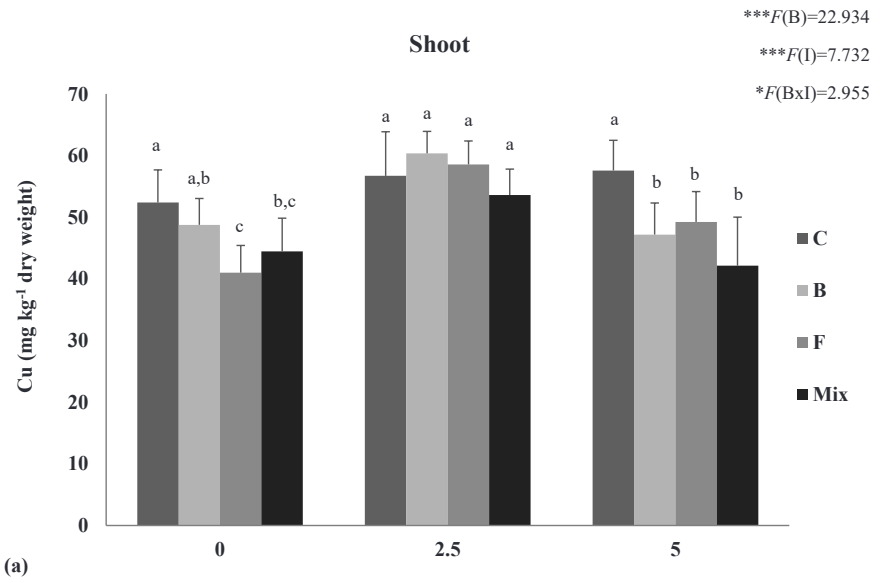
4. Discussion

Sunflower proved to be a viable and effective option for revegetating a mining soil despite the high levels of Cu present and the substantial accumulation observed in plant tissues, particularly in the roots. Indeed, the success of phytoremediation strategies in mining areas hinges on the ability of plants to tolerate high concentrations of metals, enabling the establishment of a robust vegetation cover, and sunflower demonstrated remarkable resilience and suitability for growth under these challenging conditions. This is crucial not only for stabilizing contaminants but also for restoring ecological balance and enhancing biodiversity.

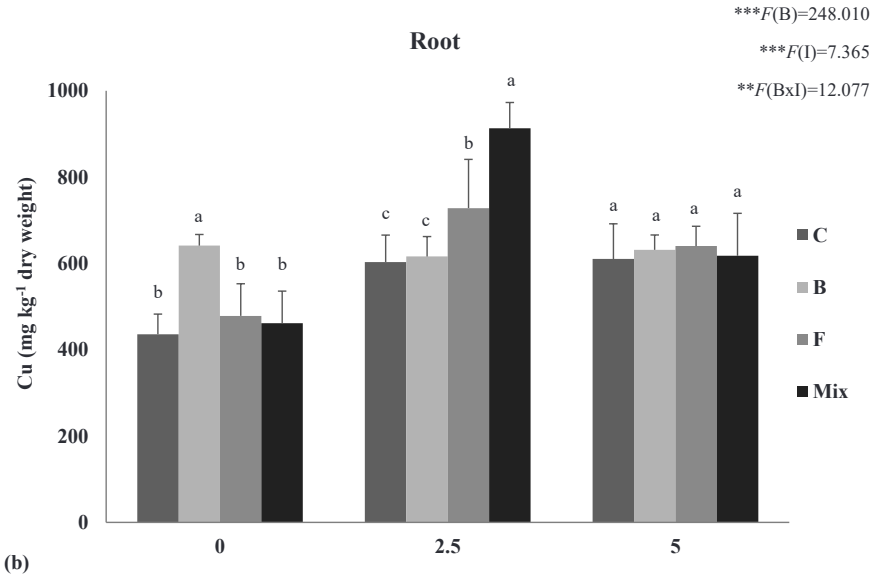
The mining soil collected from the Borralha mine exhibited a high pseudo-total concentration of Cu, measured at 1080.0 ± 0.50 mg kg^{-1}. This exceptionally high Cu concentration poses a significant threat to the surrounding environment and nearby populations. Indeed, Cu concentration exceeds by far the industrial land use limits of 91 mg Cu kg^{-1} established by the Canadian Soil Quality Guidelines. Furthermore, according to Dutch standards, which recommend a maximum of 36 mg Cu kg^{-1}, this soil is considered a high priority for remediation.

Several authors reported that sunflower is a high-yielding annual species with notable tolerance to large amounts of metal(loid)s, making it ideal for cultivation in derelict areas (Mench et al., 2018; Waseem et al., 2024). Microbial inoculation further enhanced sunflowers' growth, with F and Mix treatments showing the highest performance levels. This finding aligns with previous studies where the bacterial strain *P. reactans* EDP28 and the AMF *R. irregularis* acted as growth-promoting bioinoculants for maize in Zn- and Cd-contaminated mining soils (Moreira et al., 2016a, 2016b). These microbial inoculants have consistently demonstrated superior efficacy, likely due to their synergistic beneficial effects on plant growth and soil remediation. This is supported by the enhanced chlorophyll content of inoculated sunflower plants, along with improved nutrient use efficiency. In fact, a positive correlation was observed between SPAD readings, plant growth parameters, and NUE (Fig. 3). These findings reinforce the reliability and potential applicability of microbial inoculants in environmentally degraded conditions to foster plant growth and resilience. Similarly, Ju et al. (2019) reported that co-inoculation with PGPR and rhizobia significantly enhanced plant growth in Cu-contaminated soil. This improvement was attributed to higher nutrient (N, P, and K) content in alfalfa tissues and mitigation of reactive oxygen species accumulation and lipid peroxidation through the increased activity of antioxidant enzymes. Ke et al. (2021) also demonstrated that inoculation with *Bacillus* strains EhS5 and EhS7, either alone or in combination, significantly increased the biomass of ryegrass under Cu contamination. This improvement was primarily attributed to the modulation of root morphology and photosynthetic activity, as well as enhanced antioxidant enzyme activities.

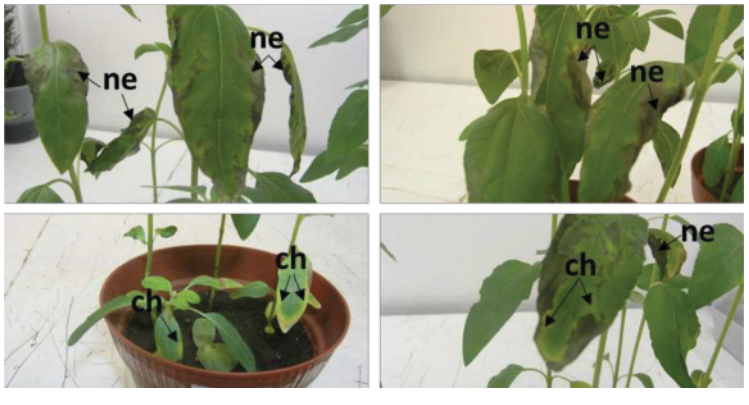
The addition of organic amendments, like biochar is often regarded as a reliable solution to ameliorate soil conditions, namely fertility, structure, water retention, and microbial activity, while promoting plant growth in mining-affected areas (Fellet et al., 2011; Li et al., 2019a; Shi et al., 2022). While lower rates of biochar are practical for large-scale field applications, in the present study, high doses of biochar were tested to assess the efficacy of its use in highly contaminated hotspots where intensive remediation is required. However, plants grown in biochar-amended soil exhibited stunted growth if compared to non-amended Cu-contaminated soil. This reduction in growth was accompanied by signs of toxicity, including yellowing leaves and dark spots (Fig. 1c). Similar symptoms were described by Bouazizi et al.



(a)



(b)



(c)

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Fig. 2. Cu accumulation (mg kg^{-1}) in shoots (a) and roots (b) of sunflower plants grown in a mining soil amended with different biochar doses (0, 2.5, and 5 %) and treated with different microbial inoculants: (C – control – non inoculated; B – rhizobacteria *P. reactans;* F – AMF *R. irregulare*; Mix – mixture of *P. reactans* and *R. irregulare*). Morphological effect of cupric stress (c) in leaves of sunflower at the end of the experiment. **ne**: necrosis and **ch**: chlorosis. Values are means ± standard deviation (n = 5). A two-way ANOVA was performed to determine the influence of biochar and of microbial inoculants on Cu accumulation in shoots and roots of sunflower plants. The results are shown with the test statistic for each case (B: biochar; I: microbial inoculants; B x I: biochar x microbial inoculants) and as NS: Non-significant at the level P > 0.05; * significant at the level P < 0.05; * * P < 0.01; * ** significant at the level P < 0.001, respectively. One-way ANOVA was performed to determine the influence of microbial inoculants on Cu accumulation in roots and shoots for each biochar dose Means for the same dose showing different letters are significantly different (P < 0.05) from each other according to Duncan test. For shoots, the F values of one-way ANOVA are *F= 5.224, NSF= 1.736 * *F= 6.057, respectively for 0, 2.5, and 5 % of biochar. For roots, the F values of one-way ANOVA are * **F= 12.418, * **F= 18.376, NSF= 0.184, respectively for 0, 2.5, and 5 % of biochar. For dry root biomass, the F values of one-way ANOVA are * **F= 14.365, * **F= 11.957, * **F= 9.402, respectively for 0, 2.5, and 5 % of biochar.

Table 2
Shoot and root Cu bioconcentration (BCF), Cu translocation factor (TF), and Cu soil available concentration (NH_4-Ac) in the different treatments (Biochar doses - 0, 2.5, and 5 %; Microbial inoculants - C – control – non inoculated; B – rhizobacteria *P. reactans;* F – AMF *R. irregulare*; Mix – mixture of *P. reactans* and *R. irregulare*).

BC	Inoculants	BCF Shoot	BCF Root	TF	NH_4-Ac (mg kg^{-1})
0	**C**	0.049 ± 0.005^{a}	0.40 ± 0.04^{b}	0.120 ± 0.018^{a}	$12.01 \pm 0.19^{a,b}$
	B	$0.045 \pm 0.004^{a,b}$	0.59 ± 0.02^{a}	0.076 ± 0.005^{b}	13.05 ± 0.29^{a}
	F	0.038 ± 0.004^{c}	0.44 ± 0.07^{b}	0.086 ± 0.007^{b}	10.88 ± 0.80^{b}
	Mix	$0.041 \pm 0.005^{b,c}$	0.43 ± 0.07^{b}	0.090 ± 0.009^{b}	11.18 ± 1.79^{b}
		*F= 5.224	* **F= 12.418	* **F= 16.169	*F= 4.767
2.5	**C**	0.053 ± 0.007^{a}	0.56 ± 0.06^{c}	$0.094 \pm 0.008^{a,b}$	18.95 ± 1.15^{b}
	B	0.056 ± 0.003^{a}	0.57 ± 0.04^{c}	0.098 ± 0.010^{b}	20.03 ± 1.08^{b}
	F	0.054 ± 0.004^{a}	0.67 ± 0.10^{b}	0.082 ± 0.014^{b}	19.73 ± 0.91^{b}
	Mix	0.050 ± 0.004^{a}	0.85 ± 0.06^{a}	0.059 ± 0.007^{c}	21.53 ± 1.08^{a}
		NSF= 1.736	* **F= 18.376	* **F= 16.124	*F= 5.213
5	**C**	0.053 ± 0.005^{a}	0.57 ± 0.08^{a}	0.095 ± 0.006^{a}	21.10 ± 0.68^{a}
	B	0.044 ± 0.005^{b}	0.58 ± 0.03^{a}	0.075 ± 0.005^{b}	21.40 ± 1.32^{a}
	F	0.046 ± 0.005^{b}	0.59 ± 0.04^{a}	0.077 ± 0.004^{b}	18.80 ± 0.89^{b}
	Mix	0.039 ± 0.007^{b}	0.57 ± 0.09^{a}	0.069 ± 0.012^{b}	16.41 ± 1.34^{c}
		* *F= 6.057	*NSF*= 0.184	* **F= 11.779	* *F= 22.426
		* **F(B)= 22.934	* **F(B)= 48.010	* **F(B)= 12.332	* **F(B)= 384.459
		* **F(I)= 7.323	* **F(I)= 7.366	* **F(I)= 28.332	* **F(I)= 9.561
		*F(BxI)= 2.955	* **F(BxI)= 12.077	* **F(BxI)= 8.738	* **F(BxI)= 12.210

Values are means ± standard deviation (n = 4). A two-way ANOVA was performed to determine the influence of biochar and of microbial inoculants on shoot and root bioconcentration and translocation factors. The results are shown with the test statistic for each case (B: biochar; I: microbial inoculants; B x I: biochar x microbial inoculants) and as NS: Non significant at the level P > 0.05; * significant at the level P < 0.05; * * P < 0.01; * ** significant at the level P < 0.001, respectively. One-way ANOVA was performed to determine the influence of microbial inoculants on shoot and root bioconcentration and translocation factors, and on Cu available concentrations in soil for each biochar dose at the end of the experiment. Means for the same biochar dose showing different letters are significantly different from each other (P < 0.05) according to Duncan test.

Table 3
Nitrogen Use Efficiency (NUE) and Phosphorus Use Efficiency (PUE) in sunflower plants grown in a mining soil amended with different biochar doses (0, 2.5, and 5 %) and treated with different microbial inoculants: (C – control – non inoculated; B – rhizobacteria *P. reactans;* F – AMF *R. irregulare*; Mix – mixture of *P. reactans* and *R. irregulare*).

	Nitrogen Use Efficiency (NUE)				Phosphorous Use Efficiency (PUE)		
	Biochar (%)				**Biochar (%)**		
Inocula	**0**	**2.5**	**5**	**Inocula**	**0**	**2.5**	**5**
C	831.3 ± 40.5^{c}	918.6 ± 67.5^{a}	999.9 ± 58.6^{a}	**C**	6719.3 ± 726.54^{b}	7439.2 ± 755.0^{b}	8315.2 ± 830.2^{c}
B	909.9 ± 86.7^{b}	926.8 ± 54.8^{a}	1046.9 ± 99.1^{a}	**B**	8561.0 ± 361.9^{a}	8494.7 ± 282.7^{a}	$10416.9 \pm 652.4^{a,b}$
F	920.3 ± 35.2^{b}	868.7 ± 53.1^{a}	1106.2 ± 34.2^{a}	**F**	8336.0 ± 478.9^{a}	8492.9 ± 772.3^{a}	9781.3 ± 278.2^{b}
Mix	1223.3 ± 34.0^{a}	961.6 ± 92.8^{a}	1044.1 ± 43.4^{a}	**Mix**	8487.9 ± 853.6^{a}	8966.2 ± 717.2^{a}	11171.1 ± 1068.7^{a}
	* **F= 51.709	NSF= 1.550	NSF= 2.334		* **F= 9.497	*F= 4.735	* **F= 12.586
	* **F(B)= 22.053				* **F(B)= 43.202		
	* **F(I)= 17.787				* **F(I)= 25.115		
	* **F(BxI)= 12.390				NSF(BxI)= 1.379		

Values are means ± standard deviation (n = 4). A two-way ANOVA was performed to determine the influence of biochar and of microbial inoculants on NUE and PUE. The results are shown with the test statistic for each case (B: biochar; I: microbial inoculants; B x I: biochar x microbial inoculants) and as NS: Non-significant at the level P > 0.05; * significant at the level P < 0.05; * * P < 0.01; * ** significant at the level P < 0.001, respectively. One-way ANOVA was performed to determine the influence of microbial inoculants on NUE and PUE for each biochar dose at the end of the experiment. Means for the same dose showing different letters are significantly different from each other (P < 0.05) according to Duncan test.

(2010) in *Phaseolus vulgaris* due to cupric stress. In this study, it was observed a significant increase in Cu accumulation and BCF in the shoots and roots of sunflowers grown in biochar-amended soils. The correlation matrix supports these findings, showing a strong negative correlation between plant growth parameters (e.g., biomass) and Cu accumulation in roots and shoots (Fig. 3). Indeed, Cu accumulation in sunflower roots was notably high exceeding the recommended limits for Cu in plants, which range from 2 to 20 mg kg^{-1} (Kabata-Pendias and Pendias, 2001). This excessive accumulation likely contributed to the weakness observed in the root systems of biochar-amended plants. Typically, Cu toxicity severely impacts roots by disrupting the root epidermis and exodermis, reducing the proliferation of root hairs, and causing severe deformation of root structure (Chen et al., 2022). While higher Cu accumulation in sunflower roots can be beneficial for remediation

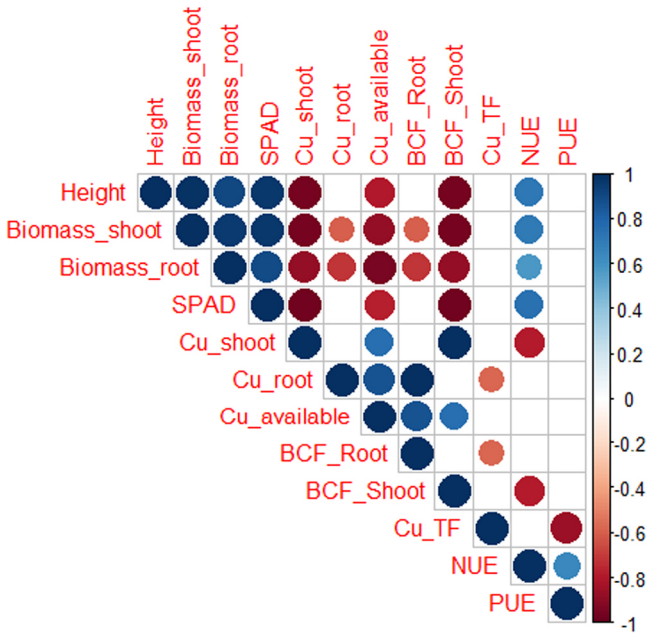

Fig. 3. Correlogram of plant parameters and Cu concentrations in plant tissues and soil. Correlations with p-values > 0.05 are considered insignificant and the correlation coefficient values are left blank. Positive correlations are displayed in blue and negative correlations are displayed in red. Cu_shoot: Cu accumulation in shoots; Cu_root: Cu accumulation in roots; Cu_available: bioavailable Cu in soil; BCF_shoot: bioconcentration factor in shoots: BCF_root: bioconcentration factor in roots: Cu_TF: translocation factor; NUE: nitrogen use efficiency; PUE: phosphorous use efficiency.

strategies by immobilizing metals and reducing their bioavailability, caution is needed if Cu toxic levels for the plant are reached. This is especially relevant in phytomanagement strategies, where plants are not only used to remediate contaminated soils but also to produce biomass for energy or economic purposes. Excessive Cu accumulation can limit the viability of biomass for applications like biofuel production or industrial use due to contamination risks. To avoid toxicity, soil amendments such as biochar must be carefully selected and applied to balance Cu bioavailability, ensuring plant health and maximizing their utility.

The excessive Cu accumulation in sunflower tissues is likely due to the increased Cu bioavailability in soil resulting from biochar application, as evidenced by the strong positive correlations between Cu availability in the soil and Cu accumulation in both roots and shoots.

These results did not follow the mainstream trend, as several studies have shown opposite results, reporting that biochar incorporation in Cu-contaminated soils significantly reduces Cu bioavailability, preventing its uptake by plants, being often considered a good mitigation strategy for increasing plant growth under Cu stress (Zhang et al., 2021; Abideen et al., 2023; Lv et al., 2024). However, our findings suggest that in this specific context, biochar may have altered soil chemistry, increased Cu availability and consequently its uptake by sunflower plants. Similarly, Jun et al. (2020) showed that biochar stimulated the uptake and accumulation of Pb, Cd, and As in the sunflower shoots. These results provide important insights into the complexities of biochar-soil interactions, challenging the assumption that biochar consistently reduces Cu bioavailability. While biochar is often a promising amendment for mitigating metal stress, these findings emphasize the need for a deeper approach, considering biochar and soil properties, the specific plant species involved, and the application context to achieve the desired outcomes. For instance, in a study with tobacco, Zhang et al. (2021) reported that in acidic soil, Cd availability and uptake increased when biochar rate exceeded 15 Mg ha^{-1}, whereas, in neutral soil, Cd levels only significantly increased at the rate of 40 Mg ha^{-1}. This reinforces that Cd uptake can vary with soil properties and biochar application rate. Jun et al. (2020) also reported that while 5 % of biochar enhanced sunflower growth in a metal-contaminated mining soil, 10 % of biochar inhibited plant growth. They attributed this inhibition to reduced soil aeration at higher biochar doses, which impaired the metabolism of sunflower roots, explaining why 10 % of biochar resulted in decreased biomass growth in sunflower plants.

However, despite the negative effect of biochar on sunflower growth, the microbial inoculants successfully mitigated this impact. In fact, microbial inoculation slightly decreased Cu availability, which could be attributed to microbial processes that immobilize or bind Cu in the soil, reducing its extractability with NH_4-Ac. On the other hand, the bacterial strain EDP28 possesses several growth-promoting traits, including high ACC-deaminase activity (Pereira et al., 2015), especially under metal-stress conditions (Moreira et al., 2016a). This trait helps plants to cope with metal stress by reducing ethylene levels, thereby alleviating stress responses. Inoculated sunflower plants exhibited enhanced chlorophyll content and improved phosphorus use efficiency (PUE). The increase in chlorophyll content suggests better photosynthetic activity, which is crucial for overall plant health and growth, while improved PUE indicates that the plants were able to utilize available phosphorus more effectively, which is vital for photosynthesis, and nutrient acquisition and transport within the plant (Khan et al., 2023). These enhancements contribute to better growth and resilience in sunflowers, even under the challenging conditions imposed by high levels of Cu and the presence of biochar.

5. Conclusions

The present study highlights the contrasting impacts of biochar and microbial inoculants on sunflower growth and metal uptake in a Cu-contaminated mining soil. Biochar significantly reduced sunflower biomass due to increased Cu availability in the soil, leading to higher accumulation in plant tissues, especially in the roots. Conversely, microbial inoculation, particularly the synergetic effect of AMF and PGPB, promoted sunflower growth and mitigated the negative effects of biochar, by improving chlorophyll content and nutrient use efficiency.

The integration of sunflowers with microbial inoculants represents a viable approach for revegetating mining areas. However, the use of biochar should be approached with caution, considering its complex interactions with soil properties and plant health. Future research should prioritize optimizing the application of microbial inoculants and biochar to enhance sustainable phytoremediation in metal-contaminated soils. Additionally, the economic viability of applying high biochar rates in real-world scenarios, particularly in targeted applications, should be assessed. Introducing incentive mechanisms, such as carbon credits, could help offset the costs of higher application rates by rewarding carbon sequestration, making these approaches more cost-effective and encouraging wider adoption.

Ethics approval

Not applicable.

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

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CRediT authorship contribution statement

Sofia Isabel Almeida Pereira: Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Paula Castro:** Writing – review & editing, Supervision, Funding acquisition. **Helena Moreira:** Writing – review & editing, Methodology, Conceptualization. **Mariana Godinho:** Writing – review & editing, Methodology, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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