PHYTOMANAGEMENT OF ZN AND CD CONTAMINATED SOIL: HELIANTHUS ANNUUS BIOMASS PRODUCTION AND METAL REMEDIATION ABILITIES WITH PLANT GROWTH PROMOTING MICROBIOTA ASSISTANCE

Ana P.G.C. Marques¹, Ana Paulo¹, Nídia S. Caetano², and Paula M.L. Castro¹

¹Universidade Catolica Portuguesa Centro de Biotecnologia e Quimica Fina
²Universidade do Porto Laboratorio de Engenharia de Processos Ambiente Biotecnologia e Energia

April 12, 2023

Abstract

Mining and industrial activity are contributing to the increase of heavy metal (HM) pollution in the environment, especially in soil. These metals leach into water, spread to plants and enter the food chain. Phytoremediation coupled to selected rhizosphere microbiota is an environmentally friendly technology designed to promote HM bioremediation in soils. In this study, sunflower (Helianthus annuus L.) was used together with Rhizophaqus irregularis, an arbuscular mycorrhizal fungi (AMF), and Cupriavidus sp. strain 1C2, a plant growth promoting rhizobacteria (PGPR), as a phytoremediation strategy to remove Zn and Cd from an industrial soil (599 mg Zn kg⁻¹ and 1.2 mg Cd kg⁻¹) and produce plant biomass - an agricultural soil was also used to obtain a H. annuus growth and metal accumulation control. The H. annuus biomass in the contaminated industrial soil was 17% lower, at harvest than that in an agricultural soil. Removals of ca. 0.04 and 0.91% of Zn and Cd respectively were obtained with the biomass produced in the industrial soil in a single crop. Bioaccumulation, remediation and translocation factors corroborated the higher Zn and Cd accumulation in the roots, compared to other plants parts. The survival of applied microbiota was indicated by a high root colonization rate of AMF and identification of strain 1C2 in the rhizosphere at the end of the phytoremediation assay. Changes in the bacterial community occurred in the industrial soil and were possibly associated to the phytoremediation effect on the rhizosphere: metals removal by the plant together with the synergic relationships established between AMF, PGPR and the autochthonous microbial community might have favoured specific soil bacterial genera, namely Nitrospira, Acidobacterium and Candidatus Koribacter. In this study, an optimized phytomagement strategy applied to a real contaminated soil was successfully tested, and plant biomass with potential for upstream energetic valorisation purposes was produced.
3 ALiCE – Associate Laboratory in Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

4 CIETI/ISEP (School of Engineering, Polytechnic of Porto), Rua Dr. António Bernardino de Almeida 431, 4249-015 Porto, Portugal

Corresponding author: amarques@ucp.pt, +351225580001

Abstract

Mining and industrial activity are contributing to the increase of heavy metal (HM) pollution in the environment, especially in soil. These metals leach into water, spread to plants and enter the food chain. Phytoremediation coupled to selected rhizosphere microbiota is an environmentally friendly technology designed to promote HM bioremediation in soils. In this study, sunflower (Helianthus annuus L.) was used together with Rhizophagus irregularis, an arbuscular mycorrhizal fungi (AMF), and Cupriavidus sp. strain 1C2, a plant growth promoting rhizobacteria (PGPR), as a phytoremediation strategy to remove Zn and Cd from an industrial soil (599 mg Zn kg\(^{-1}\) and 1.2 mg Cd kg\(^{-1}\)) and produce plant biomass - an agricultural soil was also used to obtain a H. annuus growth and metal accumulation control. The H. annuus biomass in the contaminated industrial soil was 17% lower, at harvest than that in an agricultural soil. Removals of ca. 0.04 and 0.91% of Zn and Cd respectively were obtained with the biomass produced in the industrial soil in a single crop. Bioaccumulation, remediation and translocation factors corroborated the higher Zn and Cd accumulation in the roots, compared to other plants parts. The survival of applied microbiota was indicated by a high root colonization rate of AMF and identification of strain 1C2 in the rhizosphere at the end of the phytoremediation assay. Changes in the bacterial community occurred in the industrial soil and were possibly associated to the phytoremediation effect on the rhizosphere: metals removal by the plant together with the synergetic relationships established between AMF, PGPR and the autochthonous microbial community might have favoured specific soil bacterial genera, namely Nitrospira, Acidobacterium and Candidatus Koribacter. In this study, an optimized phytomanagement strategy applied to a real contaminated soil was successfully tested, and plant biomass with potential for upstream energetic valorisation purposes was produced.

Keywords: Soil phytoremediation, plant growth promoting bacteria, arbuscular mycorrhizal fungi, sunflower, heavy metals, phytomanagement

Funding sources

FCT - Fundação para a Ciência e a Tecnologia

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.

1. Introduction

Soil is a vital resource for living organisms and the sustainability of our species highly depends on it. Nevertheless, soil contamination has been severely rising since the industrial revolution and there are currently more than 235 million hectares of distressed land all over the world (Bermudez et al., 2012), with the EU accounting for more than 3.6 million contaminated locations (FAO, 2018), the USA with 0.6 million brownfields (Shah and Daverey, 2020), while developing countries have enormous unaccounted soil polluted areas (Shah and Daverey, 2020).

Heavy metal (HM) pollution is of great distress as these compounds are not degradable but only exchangeable from one matrix to another. The area of land contaminated with HM has been growing dramatically, primarily due to mining and metallurgical production, but also due to the burning of fossil fuels, to the application of fertilisers and pesticides, and to the discharge of urban and industrial wastes and sewages (Yan et al., 2020). These compounds are severely toxic to life, many of them at very low concentrations, having deleterious effects on microbial diversity of the soil itself (Chen et al., 2014), on plant establishment and growth (Xu et al., 2018) and further affecting the food chain, and consequently animals and humans (Ahmad et al., 2011).
Numerous technologies for soil remediation have been developed based on physical, chemical or thermal procedures – which can be very expensive, ecosystem disturbing and not in line with the sustainability expected for an environmentally focused intervention (Marques et al., 2009b). Phytoremediation, a biologically based technology that uses plants and associated rhizosphere microbiota to remove or immobilize HM from contaminated soils (Marques et al., 2009b), is attracting attention – ca. 50% of papers concerning the subject have been published recently (Shah and Daverey, 2020) – as it preserves soil and the installed ecosystems, with some successful reports of field application (Reddy et al., 2019; Willscher et al., 2013). Amongst these studies, the term phytomanagement – producing revenues from biomass growing in contaminated land (Robinson et al., 2009) – is emerging as a way to increase the sustainability of the technique. Besides promoting other advantages, such as soil erosion control and clean-up, and maintaining biodiversity (Agnello et al., 2018), the establishment of a vegetation cover allows for additional profit through the use of the originated biomass for energetic, fodder or amendment purposes (Evangelou and Deram, 2014). The use of energy crops simultaneously tolerant to metal species are appropriate for applying such kind of strategy. Sunflower (Helianthus annuus L.) – an annual plant belonging to the family of Asteraceae – is a good candidate, as it is not only a well-known and widely used energy crop, but also possesses phytoremediation capability (Marques et al., 2013).

Research has indicated that the inoculation with efficient microbes to the soil ecosystem, not only improves soil quality, and consequently crop yield and quality (Singh et al., 2011), but also promotes the efficiency in the phytoremediation capacities of the selected plant species (Ma et al., 2016). Rhizospheric microorganisms such as arbuscular mycorrhizal fungi (AMF) have been successfully used in phytoremediation of HM polluted soils (Marques et al., 2009b; Moreira et al., 2016; Zhang et al., 2019). AMF form symbiotic associations with most terrestrial plant species, and are able to survive the exposure to very high levels of HM (Sánchez-Castro et al., 2017). Mycorrhized plants generally present improved nutrition and consequently higher biomass production rates (Read and Smith, 2008) and AMF can protect their hosts against HM toxicity by increasing root absorption surface areas (Davies et al., 2001) and regulating the interface between metals in the soils and plant roots (Meier et al., 2012). Plant growth promoting rhizobacteria (PGPR) have also been used for improving phytoremediation strategies with success (Jin et al., 2019), and have been described not only for their valuable growth promotion characteristics, but also as being able to decrease plant stress under harsh abiotic conditions (Glick, 2012). These rhizospheric microorganisms are able to improve plant’s nutrient procurement through nutrient solubilisation, stimulate plant growth through the synthesis of indole-3-acetic acid (IAA), decrease susceptibility to pathogens through the release of antibiotics, and diminish plants’ stress related hormones by producing specific enzymes (Glick, 2012). The possibility of promoting plant growth and survival to different abiotic stresses using a mixed microbiota, composed of PGPR and AMF, has shown to have a great potential, due to the symbiotic relationships established between AMF, PGPR, soil microbiome and the plant (Moreira et al., 2016; Pereira et al., 2016).

The aim of this work was to evaluate the possibility of producing high energetic value biomass in baren contaminated and with no further use soils. This involved the assessment of differences in soil status and on the establishment, biomass production yield and HM accumulation abilities of H. annuus in an industrial soil contaminated with zinc (Zn) and cadmium (Cd), with the assistance of selected AMF and PGPR mixed inocula, using the growth of H. annuus in an agricultural soil as a comparison. The ultimate objective is to understand if this phytomanagement approach serves the needs of biomass production for the generation of several energy products, hypothesis which is more thoroughly further on described in the report of Paulo et al. (2023).

2. Materials and Methods

2.1. Experimental design and plant growth

The soils used in this study were an agricultural soil and an industrial soil from Estarreja, both from the North of Portugal (soil properties are shown in Table 1). The industrial site has a long history of metal contamination, as for many years the discharge of solid residues and of the industrial wastewaters directly into the ground induced a HM contamination scenario (Marques et al., 2009a). Soil was collected randomly
in the selected area, to a 40 cm depth. According to Kabata Pendias and Pendias (1992) the usual total metal levels in non-contaminated soils is below 1 mg Cd kg\(^{-1}\) and 300 mg Zn kg\(^{-1}\). The agricultural soil used in the present work is within this range, while the industrial soil presents levels higher than the thresholds established. Therefore, the agricultural soil was used as control soil, representing the optimal conditions required for \textit{H. annuus} growth.

The experiment consisted of a design with 2 soil conditions (A - agricultural soil; I - industrialized soil inoculated with plant growth promoting microbiota). Each condition consisted of a 1 m\(^3\) pot filled with 1 ton of the corresponding soil.

A mixture of \textit{Rhizophagus irregularis}, an arbuscular mycorrhizal fungi (AMF) from a commercial product used in previous studies (Moreira et al., 2016; Pereira et al., 2016), and of the bacterial isolate \textit{Cupriavidus} sp. strain 1C2, a plant growth promoting rhizobacteria (PGPR) (Pereira et al., 2015), was used as plant growth promoting microbiota. Ten liter of the commercially AMF product (INOQ, GmbH, Germany), was mixed in the Estarreja’s soil before seedling. The same volume of sterilised vermiculite was added to the agricultural soil. The bacterial strain 1C2 was grown overnight at 150 rpm and 30 °C in Luria-Bertani’s (LB) medium. The pellets were then washed twice and re-suspended in 10 mM phosphate buffer pH 8.0 to get an inoculum density of ca. 10\(^{8}\) CFU ml\(^{-1}\). After seedling, the industrial soil was inoculated with 2 L of bacterial solution, while 2 L of sterilized phosphate buffer was added to the agricultural soil.

\textit{H. annuus} seeds (variety IBERICO, LusoSem, Portugal) were surface sterilized with 0.5% (v/v) NaOCl for 10 min and were subsequently washed with sterilized deionized water. All seeds were sowed directly in the tested soils, and after seedling were reduced to 100 per pot (at a distance of 10x10 cm).

2.2. Plants biomass and metal accumulation monitoring

\textit{H. annuus} plants were harvested after 120 days, subdivided in root, stems (stems and leaves were not separated and are considered in this portion), flowers and seeds and washed with deionized water, followed by HCl 0.1 M. Samples were air dried and seeds were extracted, then remaining tissues were oven dried at 70 °C for 48 h, after which the biomass dry weight was measured. Plant samples were then ground and digested at high temperature in a PerkinElmer MicroWave 3000 following the 3052 USEPA method. Cadmium and Zn content was determined using Flame Atomization - Atomic Absorbance Spectrometry (FA-AAS) of the digests (Wallinga et al., 1989) in a Unicam 960 spectrophotometer (Waltham, USA).

2.3. Bioaccumulation factor and remediation ratio

Bioaccumulation (or bioconcentration) factors (BF) and remediation ratios (RR) were calculated according to Mani et al. (2015), and translocation factors (TF) according to Moreira et al. (2014), as shown in eq. (1) to (3):

\[
BF = \frac{\text{Metal concentration in the aboveground tissues}}{\text{Metal concentration in the soil}}
\]

\[
TF = \frac{\text{Metal concentration in the aboveground tissues}}{\text{Metal concentration in the root tissues}}
\]

\[
RR = \frac{\text{Metal concentration in the aboveground tissues} \times \text{Plant dry aboveground biomass}}{\text{Metal concentration in the soil} \times \text{Soil dry weight in the container}}
\]

BF, TF and RR were also calculated for the agricultural soil in order to also have information on the pattern of Zn and Cd uptake by the plant, when these metals are also present in the soil at lower concentrations.

2.4. Cd and Zn mobilization in soil

Soil of each pot was collected at the beginning and at the end of the experiment in order to assess the water and the ammonium acetate (NH\(_4\)-Ac) extractable Cd and Zn fractions. Solutions of 1:5 soil water and 1:5 soil NH\(_4\)-Ac (de Koe, 1994) were incubated in rotating flasks at 20 °C for 2 h. The extracts were then centrifuged at 38000 rpm, for 10 min, and the supernatants filtered over a 0.45 μm cellulose acetate filter and collected in test tubes. Cd and Zn contents were determined using the FA-AAS, as described above.

2.5. Monitoring of bacterial community in soils
DNeasy PowerSoil Kit (Qiagen, Germany) was used for extracting DNA from duplicate soil samples, collected at the beginning and at the end of plant growth, for both soil conditions, according to manufacturer’s instructions. Qubit (Thermo Fisher Scientific, USA) was used for measuring the DNA concentration and the extracted DNA was stored at -20 °C for future use.

NGS of the extracted DNA was performed at GATC-Eurofins (Konstanz, Germany), which included DNA amplification, libraries preparation, sequencing and bioinformatics data analysis. The 16S rRNA phylogenetic gene paired-end sequencing was performed to cover the V3-V4 hypervariable region (Illumina MiSeq platform) by using two primers (357F - TACGGGAGGCAGCAG, (Turner et al., 1999); 800R – CCAGGG-TATCTAATCC, (Kisand et al., 2002). The microbiome analysis and profiling was performed as described in Paulo at al. (2021). The raw sequence data was deposited in Sequence Read Archive (SRA) from NCBI database, within the BioProject with accession number PRJNA830831. BLAST from NCBI was used (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for a more complete identification of operational taxonomic unit (OTU) sequences from bacterial families with high relative abundance but not identified up to the genus level.

2.6. Root colonisation by AMF

A sub-sample of fresh fine roots was collected from the H. annuus plants harvested from each soil condition (agricultural and industrial), and were cut into approximately 1-2 cm pieces, heated in a 80 °C water bath for 30 min in 10% (w/v) KOH, following a procedure adapted from Vierheilig et al. (1998). After heating, the KOH solution was poured out and roots were cleared by adding HCl 3% (v/v) for 10 min. The roots were then stained using a staining solution consisting of 5% ink (Pelican 4001, Brilliant black, Fountain Pen Ink) diluted in 5% (v/v) acetic acid. After this the roots were boiled for 4 min at 80 °C. Following staining, the roots were rinsed several times with tap water. Stained root samples were examined microscopically to assess the percentage of mycorrhizal colonisation using the grid-line intersect method (Giovannetti and Mosse, 1980).

2.7. Statistical Analysis

Differences in the parameters in all tested treatments were statistically analysed by one-way ANOVA and T-tests using the IBM SPSS Statistics program (IBM, Armonk, NY, USA, version 28.0). Duncan test (P<0.05) was performed to establish the significance of the differences among the means.

2.8. Chemical Reagents

The chemicals used were of analytical-grade and were obtained from Pronalab (Sintra, Portugal), Promega (USA) - liquid reagents, Sigma Aldrich (Missouri, USA) and Merck (Darmstad, Germany) - solid reagents.

3. Results

3.1. H. annuus biomass production yields

Biomass of the different plant sections at harvest was determined. H. annuus plants growing in agricultural soil always presented higher biomass yields for any plant section than plants grown on industrial soil (Table 2). Values ranged from ca. 63 g for the seeds and ca. 750 g for the stems of H. annuus plants growing in agricultural soil, and from ca. 52 g for the seeds to ca. 620 g for the stems of H. annuus plants growing in industrial soil. Biomass production was higher for the stems, followed by the flowers, the seeds and the roots, independently from soil type. Also, and as a common trait, H. annuus plants growing in agricultural soil always presented higher biomass yields for any plant section than plants grown on industrial soil. The root was more affected by the soil matrix, reducing ca. 42% in the industrial soil compared to the agricultural soil, with stems and seeds being similarly affected by soil condition, decreasing ca. 17% in the contaminated soil in comparison to the agricultural soil. The formation of flowers from plants grown in industrial soil was 10% lower compared to the agricultural soil. Considering the entire plant as a sum of all plant sections, the biomass reduction of overall H. annuus growing in the industrial soil was ca. 17%, when comparing to plants grown in the agricultural soil.
3.2. Metal accumulation in *H. annuus* plants

The effect of growing *H. annuus* plants in metal contaminated soil was assessed and levels of Zn and Cd for the different plant sections are registered in Table 3. Values for Zn and Cd were higher in plants grown in industrial soil, being also higher in roots compared to seeds, for both soil conditions. The highest accumulation of Cd and Zn was registered for the roots independently of the soil matrix and the accumulation profile was as follows: roots > stems > flowers > seeds, for both soil conditions and metals; no Cd was detected in the flowers and seeds of *H. annuus* grown in agricultural soil. Zn values ranged from an average of 2 mg kg\(^{-1}\) in seeds to 67 mg kg\(^{-1}\) in roots of plants grown in agricultural soil and from an average of 4 mg kg\(^{-1}\) in seeds to 434 mg kg\(^{-1}\) in roots of plants grown in industrial soil. For Cd, values ranged from the non-detection in seeds to 1.6 mg kg\(^{-1}\) in roots of plants grown in agricultural soil and from an average of 0.5 mg kg\(^{-1}\) in seeds to 24 mg kg\(^{-1}\) in roots of plants grown in industrial soil.

In all plant tissues, except for the seeds, significant (P < 0.05) differences were shown between the levels of Zn and Cd, with plants grown in the industrial soil presenting higher accumulation than those grown in the agricultural soil. The accumulation levels in the seeds showed no significant (P < 0.05) difference between soils. The highest accumulation of both Cd and Zn were registered for the roots, independently of the soil matrix, followed by the stems, then the roots and finally the seeds. Additionally, no Cd was detected in the flowers and seeds of *H. annuus* grown in agricultural soil.

3.3. Bioaccumulation and translocation factors and remediation ratios

Bioaccumulation factors (BF), translocation factors (TF) and remediation ratios (RR) were calculated and are represented in Figure 1. All BF, TF and RR were generally higher for Cd than for Zn. For Zn, BF was higher for plants growing in the agricultural soil (maximum of ca. 1.4), while for Cd the factors were higher for plants exposed to the industrial soil (maximum ca. 11.2). The same trend was also verified for the RRs, with the highest (ca. 9.8) and lowest (ca. 0.39) values being shown for Cd and Zn for *H. annuus* grown in industrial soil, respectively (Figure 1). For TF, values were generally lower than 1 and were maximized both for Zn and Cd in plants grown in the agricultural soil (maximums of ca. 0.76 and 1.33 respectively).

3.4. Cd and Zn mobilization in soils

No metals were detected in the water extracts at the beginning and at the end of the experiment. Metal levels in soil ammonium acetate extracts at the beginning and at the end of the experiment are described in Table 4. No bioavailable Cd was detected in any of the samples. Either at the beginning or at the end of the experiment, the levels of bioavailable Zn were always significantly (P < 0.05) higher for the industrial soil. Also, and for both tested soils, levels of bioavailable Zn increased significantly (P < 0.05) at the end of the study (Table 4).

3.5. Monitoring of bacterial community present in soils

The microbial community diversity at the phylum level was found to be similar in both soil conditions, mostly regarding the abundance of *Actinobacteria*, *Proteobacteria*, *Acidobacteria* and *Firmicutes* (Figure 2). *Actinobacteria* dominated the bacterial community in both soils, representing more than 45% and 53% of the total relative abundance in the agricultural and the industrial soil, respectively. *Proteobacteria* presented between 18 and 29% of relative abundance in both soil conditions. *Acidobacteria* were present with a similar relative abundance in agricultural soil throughout plant growth (10-16%), increasing from 4 to 10% in the industrial soil. *Firmicutes* presented similar relative abundances between both soil conditions (2-5.5%). *Gemmatimonadetes* were not identified in any of the industrial soil samples but were detected in all agricultural soil samples, increasing their relative abundance at the end of plant growth. *Nitrospirae* were present in higher relative abundance in the agricultural soil compared to the industrial soil, where their numbers increased at the end of plant growth. *Cyanobacteria* were only identified in the agricultural soil (relative abundance between 0.6 and 1.3%).

Both soil conditions presented a high relative abundance of *Actinobacteria*, *Rubrobacteria* and *Alphaproteobacteria* classes (Figure 3). *Acidobacteria* and *Nitrospira* presented higher and stable
relative abundance in the agricultural soil, compared to the industrial soil and Acidimicrobiia and Gemmatimonadetes were mostly detected in the agricultural soil while Betaproteobacteria were more abundant in the industrial soil. Thermoleophilia, Bacilli and Alphaproteobacteria were identified in similar relative abundance in both types of soil. Comparing the beginning and end of plant growth, Gemmatimonadetes increased while Betaproteobacteria decreased in the agricultural soil. In the industrial soil, both Acidobacteria and Nitrosospira increased while Actinobacteria decreased in relative abundance, at the harvest.

The ten dominant bacterial genera accounted for more than 70% of the total relative abundance in most of the soil samples (except for I2). Several bacterial genera were present with similar relative abundance in both soil conditions, at beginning and end of plant growth (Figure 4). This was the case of Gaiella, which presented the highest relative abundance in all soil samples, and Sphingomonas, Nakamuraella and Bacillus, present with similar relative abundance in both soils. However, the agricultural soil presented several bacteria with higher relative abundance compared to the industrial soil. Acidobacterium and Nitrospira were present with higher relative abundance in the agricultural soil compared to the industrial soil, as well as Solirubrobacter, Gemmatirosa, Aciditerrimonas, Gemmatimonas and Candidatus Koribacter. On the other hand, the industrial soil presented a higher relative abundance of Conexibacter, Geodermatophilus, Blastococcus and Arthrobacter bacterial genera.

Although most of the bacterial genera kept their relative abundance between the beginning and the end of plant growth, Gemmatirosa increased in relative abundance in the agricultural soil, while Acidobacterium, Nitrospira and Candidatus Koribacter increased in the industrial soil.

Ten OTU sequences belonging to Burkholderiaceae family were further identified in the industrial soil, using BLAST from NCBI. All sequences were found to be highly similar (>99% identity) to the 16S rRNA sequence of the Cupriavidus sp. 1C2 strain, inoculated at the beginning of plant growth in the industrial soil. According to this information, Cupriavidus sp. 1C2 was identified in the industrial soil, presenting a high relative abundance at the beginning and end of plant growth (between 12 and 17%). This bacterial genus was not identified in the agricultural soil.

3.6. Mycorrhizal colonisation of roots

Mycorrhizal colonisation was determined for root samples from H. annuus plants grown in both tested soils at harvest and percent colonisations are described in Table 5. Colonisation was observed for plants growing in both soil conditions, with plants growing in industrial soil (inoculated with the selected AMF Rhizophagus irregularis) showing a higher degree of colonisation (ca. 43%) when comparing to non-inoculated plants grown in agricultural soil (ca. 27%).

4. Discussion

A mycorrhizal fungi product and a bacterial isolate were used as plant growth promoting microbiota for the growth of H. annuus in the presence of Zn and Cd in an industrial soil. Nevertheless, a lower H. annuus biomass production was still observed in the industrial soil, compared to the non-contaminated agricultural soil. This is associated to the high Zn and Cd contamination level (Kabata-Pendias, 1992) which as a consequence was partly bioaccumulated by the plant. The contamination of a soil with such levels of metals can impair plant growth not only by disturbing its nutrient uptake – high concentrations of metals in the soil will result in reduced uptake of water and nutrients (Ahmad et al., 2011) – but also by damaging severely its metabolic activities, by inhibiting physiologically active enzymes and mineral metabolism and consequently decreasing the rates of photosynthesis (Alaboudi et al., 2018). In the present study, the impairment was more severe for the plant’s roots compared to the upper parts of the plants, as previously described in literature for H. annuus. For example, Alaboudi et al. (2018) reported decreases in the root and shoot dry weights of H. annuus of 35 and 14%, respectively, when exposed to a 10 mg Cd kg⁻¹ spiked soil. Marques et al. (2013) showed that root and shoot dry weights of H. annuus were reduced by up to 83 and 40%, respectively, when exposed to a 500 mg Zn kg⁻¹ spiked soil, twice higher values when compared to those obtained in the present study. Despite the greater effect in H. annuus root, the overall biomass decrease was lower than 20%. This decrease was accompanied by an increase in both Zn and Cd accumulation in all plant tissues.
maximum increases of up to ca. 6.5 and 6.1-fold were registered for the root and stems tissues for Zn, and up to 15 times for the root and stem tissues for Cd. This resulted in Zn and Cd accumulation levels exceeding the phytotoxic concentrations proposed by Kabata Pendias and Pendias (1992) – 100 to 400 mg Zn kg\(^{-1}\) and 5 mg Cd kg\(^{-1}\) – with values as high as 434 mg Zn kg\(^{-1}\) and 24 mg Cd kg\(^{-1}\). \textit{H. annuus} plants seemed to have adopted an immobilization strategy of the metals in the roots, which presented a significantly higher level of both Zn and Cd accumulation. This generally resulted in translocation factors (TF) lower than 1 – similarly to what happened in previous studies (Marques et al., 2013). The range of Zn and Cd concentrations observed in this study for the tissues of \textit{H. annuus} grown in the industrial site are within the range of other studies with similar soil contamination profiles: for a metal contaminated soil (575 mg Zn kg\(^{-1}\) and 6 mg Cd kg\(^{-1}\)) Meers et al. (2005) described levels of Zn and Cd accumulation in the shoots of \textit{H. annuus} of up to ca. 250 and 5 mg kg\(^{-1}\), respectively; Nehnevajova et al. (2009) reported concentrations of Zn and Cd in the stems of up to 274 and 0.67 mg kg\(^{-1}\), respectively, and in the leaves of up to 401 and 1.58 mg kg\(^{-1}\), respectively, for \textit{H. annuus} plants grown in a soil contaminated with average levels of 759 mg Zn kg\(^{-1}\) and 0.81 mg Cd kg\(^{-1}\). However, the values reported in these studies are converted in bioaccumulation factors (BF) lower than those observed in the present report for the industrial soil for both Cd and Zn. In this work, BFs and consequently remediation ratios (RR) were always higher for Cd than for Zn, which is in accordance with the commonly recognised higher Cd transfer coefficient between soil and plant (Kloke et al., 1984) probably affected by the positive relation between metal electro-negativity (according to the Pauling scale, Zn=1.65 and Cd=1.69) and metal uptake ability. For Zn, both BF and RR values decreased when sunflower was grown in contaminated soil and the opposite happened for Cd, nevertheless, values were generally higher for all cases than those registered in the report of Mani et al. (2015): RR up to ca. 0.6 and 0.2, and BF up to ca. 2 and 0.75, for Cd and Zn respectively, for a soil showing a contamination of 2.2 mg Cd kg\(^{-1}\) and 39 mg Zn kg\(^{-1}\).

Despite the growth reduction associated to increased metal uptake, \textit{H. annuus} plants were able to grow and produce significant amounts of biomass under the harsh conditions of metal toxicity presented by the industrial soil. Although this could be explained by different mechanisms described in literature for the plant to endure the presence of such heavy metals, such as enhancement of metal detoxification through metal sequestering molecules production (Tomas et al., 2015), improvement of antioxidant systems (Nehnevajova et al., 2012), and tolerance mechanisms implicating plant growth regulators (Tassi et al., 2008), the establishment of a symbiotic association between the plant and the mixture of beneficial microorganisms might have played an important role in \textit{H. annuus} growth and phytoremediation ability. AMF and PGPR can promote a higher resistance to toxicity and ability to grow and produce biomass under metal stress. In the present work, in order to help the plants coping with the installed metal contamination conditions, the industrial soil was inoculated with the AMF \textit{Rhizopagus irregularis} – shown in previous studies to enhance biomass production and accumulation of nutrients in \textit{H. annuus} (Pereira et al., 2016) under abiotic stress – and consequently the \textit{H. annuus} plants growing in this soil presented higher AMF colonization rates in their roots. High arbuscule formation is revealing of active AMF symbiosis as they are exchange sites in the root cortex for mineral elements (Davies et al., 2001; Read and Smith, 2008). This increase is similar to the one reported in the study by Zhang et al. (2019) when a soil from a recycling base was inoculated with the AMF \textit{Funneliformis caledonium} for assisting the growth of garlic chives and \textit{H. annuus}. Similarly, the industrial soil was inoculated with a heavy metal site derived bacterial strain \textit{Cupriavidus} sp. strain 1C2, known from previous studies to have plant growth promoting traits (Pereira et al., 2015) and to help \textit{H. annuus} cope with abiotic stress (Marques et al., 2013). From the follow up of the microbial community made throughout the experiment, it was observed that the inoculated PGPR prevailed in the rhizosphere, possibly keeping its activity until the end of the experiment, which was somehow expected, since as aforementioned this strain was initially isolated from an industrial soil contaminated with heavy metals (Pires, 2010). Although inoculating AMF or PGPR alone can improve plant performance under different abiotic stressing conditions, by helping plant’s establishment, survival, growth and physiology (Moreira et al., 2020), several studies have shown that AMF and PGPR together can provide a more positive environment for the plant and the rhizosphere microbiota (Mani et al., 2015; Moreira et al., 2016; Pereira et al., 2016). The AMF action might not only be associated with healthier plants physiological traits, but also due to its positive effect on soil bacteria:
AMF exudates can be used as source of nutrients by bacteria, while creating bacterial habitats with their hyphae, and soil microbes produce compounds that can increase the amount of root exudates as well as plant hormones, influencing in this way AMF establishment and rate of root colonization (Moreira et al., 2016; Pereira et al., 2016).

Another key strategy to increase the effectiveness of phytoremediation is to increase HM bioavailability (Yan et al., 2020), and both Cd and Zn became more bioavailable at the end of the experiment. Exudates from plant roots can influence the pH of the soil solution and consequently affect metal availability, as soil acidification on the rhizosphere is a key mechanism accountable for rising metal solubility (Luo et al., 2000). Biosurfactants and organic acids, produced by the bacterial community present in the rhizosphere, can also increase metal bioavailability, by helping the desorption of metals from soil matrix and maximizing metals chelation (Shah and Daverey, 2020). Also, arbuscular mycorrhizal fungi can alter soil properties through their metabolic processes and consequently promote changes in the availability of the existing metals (Leung et al., 2013). Some of these factors may have contributed to the rise of metal availability in both tested situations.

Cadmium and Zn being toxic metals, their presence and increased bioavailability throughout the experiments can cause changes in the composition of soil microbial communities. The functional diversity of soil microbial community has been used as a pointer for assessing the effect of revegetation on soil quality (Agnello et al., 2018) and a considerable number of studies have focused on the liaison between soil microbial communities and soil contamination by heavy metals (Agnello et al., 2018; Marques et al., 2013; Moreira et al., 2016). Despite the different use and contamination status of the tested soils, the initial bacterial phyla dominance was similar. A deeper analysis into the taxonomical classification allowed identifying a higher diversity of bacterial genera present in the agricultural compared to the industrial soil, which is in accordance to the report of de Quadros et al., (2016), which showed a decrease in the diversity of the microbial community of post-mined sites by comparison with undisturbed land, as expected, due to the lower level of abiotic stressors present in the agricultural soil. Acidobacteria, Firmicutes, Actinobacteria and Proteobacteria were found to be among the most dominant bacterial phyla present in soil (Fierer et al., 2007). The initial higher numbers of Acidobacteria, and Acidobacterium, in the agricultural soil could be explained by the fact that members of the Acidobacteriaceae family are abundant in mature soils with plant cover, in the presence of carbon, nutrients and lower pH (Ivanova et al., 2020). When looking for changes in soil microbiota it was observed that plant-soil interaction did not affect significantly the bacterial community dynamics of the agricultural soil. On the other hand, the phytoremediation process promoted a greater effect on the industrial microbial soil dynamics. Gaiella, from the Rubrobacteria class (Actinobacteria) was dominant in both soils, regardless of the land use and contamination status. Gaiellamight be ubiquitous in soil and tolerant to HMs, since it was previously identified in farmland (Liu et al., 2017) and in heavy metals contaminated soil (Hu et al., 2021). Nakamurella (Actinobacteria class and phyla) was also present in both soils. This is a more rare genus, with some species isolated from soil (Nouioui et al., 2017). Sphingomonas sp. (Alphaproteobacteria class) was also identified with high relative abundance in the tested soils. This bacterium is widely distributed in water and soil, associated to plants and can improve plants development in stress conditions such as drought, salinity and in the presence of heavy metals (Asaf et al., 2020). On the other hand, some bacteria were identified with higher relative abundance in the industrial soil, such as Arthrobacter. These bacteria are normally found in the rhizosphere, being also present in HMs contaminated sites (Gallo et al., 2019).

Ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) are sensitive to HMs and their enzyme activities can be used as bioindicators of HMs contamination (li et al., 2020). Representative AOB and dominant in soil microbial communities (Nardi et al., 2020), both Nitrosomonas and Nitrosospira bacterial genera were identified in the tested soils. The presence of a lower abundance of Nitrosospira (AOB) in the industrial compared to the agricultural soil might be associated to HMs contamination sensitivity, while the increased abundance of Nitrosospira (NOB) after the phytoremediation process might be linked to a decrease in HMs concentration in the rhizosphere. Other bacteria possibly favoured by the phytoremediation process were Acidobacterium and Candidatus Koribacter. Despite being known as HM-tolerant (Zhang et al., 2021), Gemmatimonadetes was not present in the industrial soil in relative abundance during phytoremediation.
process.

The background levels of Zn in soils should not be higher than 300 mg Zn kg\(^{-1}\) dry soil and 1 mg Cd kg\(^{-1}\) dry soil (EC Directive 86/278/CEE from June 12\(^{th}\)). The industrial soil is above the legislated limits and fell within the range of contamination seen as potential for the application of phytoremediation-based clean-up strategies. One of the disadvantages of the technique is the possible toxicity of high levels of metals present in the plants, after accumulation. Using the data presented on this study, considering equal plant development (4 crops/year) and similar soil contamination characteristics, a minimum of 20 crops would be necessary to achieve Cd soil clean-up and 1211 crops for Zn (which will correspond for 1 ton of soil to a minimum time span of 5 and more than 300 years respectively). The accumulations of Zn and Cd in sunflowers grown in the industrial site were above the range for plants cultivated on uncontaminated parcels (Chaney, 1989), and well above concentrations shown to be damaging to animals (Underwood, E.J. and Suttle, 1999) and therefore their use as products for human or livestock consumption is not allowed. Alternative applications for this biomass should be thus considered, namely as sources of biomass for energy production (Marques et al., 2020). This option has emerged as a possibility in recent reports (Agnello et al., 2018; Balsamo et al., 2015) but needs further study, as heavy metals may affect the product generation routes and ultimately contamination levels should always be quantified to understand if the final product is compatible with the anticipated purpose (Agnello et al., 2018).

The assessment of the potential of the *H. annuus* biomass here produced for the generation of biofuels, namely using an integrated strategy encompassing the utilization of all the plant sections and analysis of the resulting products concerning heavy metals contents, is further analysed by the study of Paulo et al. (2023). This report shows that the production of biofuels (namely oil, bioethanol, biodiesel and biogas) from the biomass derived from such phytomanagement strategy of this industrial HM contaminated soil can be feasible and can serve to counterpart the growing need for biomass for biofuels generation.

Conclusions

The total *H. annuus* biomass obtained after the phytoremediation strategy was less than 20% lower compared to growth in a control soil. The highest metal accumulation occurred in the plant’s roots followed by the stems, flowers and seeds, associated to low translocation factors. An expected increase in Cd and Zn mobilization in soil was also verified. The persistence of inoculated PGPB and AMF colonization in the treated industrial soil was observed, indicating the ability of both types of microorganisms to survive and play a role in plants health protection during growth and metal uptake activity.

An upgraded phytoremediation strategy based on the synergetic action of different plant growth promoting microbiota with and energetic crop can lead to the production of a significant amount of biomass, increasing its potential use for different valorisation purposes.

Acknowledgements

This work was financially supported by: project PHYTOENERGY-PTDC/BTA-BTA/28761/2017 (POCI-01-0145-FEDER-028761), funded by National Funds from FCT - Fundacao para a Ciencia e a Tecnologia; and UIDB/50016/2020 (CBQF), LA/P/0045/2020 (ALiCE), UIDB/00511/2020 and UIDP/00511/2020 (LEP-ABE), and UIDB/04730/2020 (CIETI), funded by national funds through FCT/MCTES (PIDDAC).

References


Paulo AMS, Caetano NS, Marques APGC. Assessment of the potential of sunflower grown in metal-contaminated soils for the production of biofuels. 2023 (submitted)


Hosted file


Hosted file