Universidade de Aveiro Departamento de Biologia
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Bioinoculants and biochar for sunflower growth promotion in a mining soil

Bioinoculantes e biochar na promoção de crescimento de girassol em solos de minas

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Sofia Isabel Almeida Pereira, Investigadora da Escola Superior de Biotecnologia da Universidade Católica Portuguesa e coorientação da Doutora Isabel da Silva Henriques, Professora Auxiliar, Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra.

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Dedico este trabalho aos meus pais, irmã e ao meu fiel e eterno companheiro, Bunny.

o júri

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palavras-chave Girassol, Metais, Biochar, PGPR, AMF, Fitorremediação.

resumo

A contaminação dos solos é uma preocupação global dos tempos atuais, devido à contribuição de numerosas atividades antropogénicas como a exploração mineira. A remoção de resíduos mineiros e a consequente deterioração das propriedades do solo, pode causar vários problemas ambientais e de saúde, levando à contaminação de extensas áreas com metais. A mina da Borralha até a sua desativação no século passado foi um dos maiores produtores de volfrâmio. Plantas com valor energético, como o girassol podem conferir um valor acrescentado a esta área, uma vez que os solos deste local possuem altos níveis de metais. Assim, o projeto PhytoSUDOE pretende restaurar estes locais contaminados através da implementação de técnicas de fitorremediação, de forma a estimular a funcionalidade do ecossistema. Inóculos microbianos como rizobactérias promotoras do crescimento de plantas (sigla inglesa PGPR) e fungos micorrízicos arbusculares (sigla inglesa AMF) podem melhorar a eficiência da fitorremediação, através da promoção do crescimento de plantas quando expostas a condições adversas (e.g. contaminação com metais). Por outro lado, a aplicação de fertilizantes orgânicos como o biochar (BC) pode influenciar o pH do solo, a retenção de água e a manutenção de nutrientes. Pela sua capacidade adsortiva em relação aos metais, podem ainda reduzir os níveis destes contaminantes e a consequente biodisponibilidade nos solos. O principal objetivo deste trabalho foi avaliar o potencial do BC e dos inoculantes microbianos para atuarem como auxiliadores da fitorremediação, para a promoção do crescimento de girassol num solo mineiro contaminado com metais. As sementes de girassol foram inoculadas com a bactéria *Pseudomonas reactans (B)*, o AMF comercial (F) e com uma mistura de *P. reactans* e AMF (Mix) e semeadas num solo mineiro suplementado com quatro percentagens de BC (0, 2.5, 5 e 10% (m/m)). O aumento da concentração de BC induziu uma redução generalizada nos parâmetros biométricos da planta, contudo a inoculação teve uma influência positiva nestes parâmetros, particularmente a inoculação com F e Mix, uma vez que aumentaram significativamente a produção de biomassa e absorção equilibrada dos nutrientes, reduzindo desta forma os efeitos nocivos dos metais no crescimento de girassol. A acumulação de Cu nos tecidos vegetais foi, em geral, mais alta nas raízes do que na parte aérea. A adição de BC a 2.5 e 5% resultou em aumentos médios de 28 e 29% respetivamente, para o conteúdo em Cu nas raízes do girassol. Contudo, os níveis de acumulação de Zn foram maiores na parte aérea do que na raiz desta planta. O conteúdo em azoto e fósforo nos tecidos vegetais foi geralmente maior na parte aérea do que nas raízes. As comunidades bacterianas presentes nas amostras rizosféricas foram analisadas pela amplificação do gene 16S rRNA e separação dos fragmentos por DGGE (Eletroforese em gel de gradiente desnaturante). Em geral, a comunidade bacteriana variou de acordo com o inoculante microbiano aplicado, onde a inoculação com AMF aparentou ter uma maior influência na comunidade microbiana do solo. Este trabalho demonstrou o potencial da combinação do BC e dos inóculos microbianos de formar a promover o crescimento do girassol em solos contaminados com metais e o seu potencial para a implementação de diferentes estratégias de fitogestão.

keywords Sunflower, Metals, Biochar, PGPR, AMF, Phytoremediation.

abstract

Soil contamination is a present-day worldwide concern due to the contribution of numerous anthropogenic activities, such as mining activities. The disposal of mine tailings along with deterioration of soil properties can generate several environmental and health problems, thus leading to metal contamination of extensive areas. Borralha mine was one of the biggest producers of tungsten in the past century, until its deactivation. Energy crops, such as sunflower can grant added value to this area since it integrates soils with high levels of metals. Therefore, PhytoSUDOE project intends to restore these contaminated sites, through the implementation of phytoremediation techniques, in order to stimulate ecosystem functionality. Microbial inoculants such as Plant Growth Promoting Rhizobacteria (PGPR) and Arbuscular Mycorrhizal Fungi (AMF) can enhance phytoremediation efficiency through enhanced plant growth, when exposed to stress conditions (e.g. metal contamination). On the other hand, the application of organic soil amendments, like biochar (BC) can influence soil pH, water retention, and nutrient maintenance. Through its adsorption capacity towards metals, it can also reduce the levels of these contaminants and consequent bioavailability in contaminated soil. The main objective of this work was to evaluate the potential of BC amendment and application of microbial inoculants to perform as phytoremediation assistants, for plant growth promotion, specifically for sunflower plants grown in a mining metal-contaminated soil. Sunflower seedlings were inoculated with the bacteria *Pseudomonas reactans* (B), a commercial AMF (F) and with a mixture of *P. reactans* and AMF (Mix) grown in a mine soil amended with four percentages of BC (0, 2.5, 5 e 10% (w/w)). Increasing BC levels induced a generic reduction of plant biometric parameters, although inoculation (particularly F and mixed inoculation) had a positive influence on these parameters, since they increased significantly biomass production and balanced nutrient uptake, thus reducing the harmful effects of metals on sunflower growth. Cu accumulation in plant tissues was generally higher in roots than in shoots. BC addition at 2.5 and 5% induced average increases of 28 and 29% respectively, when in comparison to Cu content in roots. However, higher levels of Zn were recorded on sunflower shoots than on roots, as well as N and P contents. The bacterial communities present in rhizospheric samples was analyzed by amplifying 16S rRNA gene fragments, which were separated by DGGE (Denaturing gradient gel electrophoresis). In general, the bacterial communities varied in accordance with the microbial inoculant, where the AMF inoculation appeared to have a higher influence on the bacterial soil communities. This work demonstrates the potential of combining BC and bioinoculants in order to promote sunflower growth in metal contaminated soils and their potential for implementing different phytomanagement strategies.

Contents

List of figures

List of tables

Abbreviations

°C - celsius degree
- OH - hydroxyl group
Al- aluminium
ACC - 1-aminocyclopropane-1-carboxylic acid
AMF - arbuscular mycorrhizal fungi
As - arsenic
B - bacterial inoculation
BC - biochar
BCF - bioconcentration factor
cm - centimeter
cm^3 - cubic centimeter
C - control
CaO - calcium oxide
$CaWO_4$ - scheelite
Cd - cadmium
CFU - colony forming unit
Co - cobalt
CO_2 - carbon dioxide
Cr- chromium
Cu - copper
D - simpson diversity index
DNA - deoxyribonucleic acid
DGGE - denaturing gradient gel electrophoresis
E - equitability
EDTA - ethylenediamine tetraacetic acid
ESB - UCP - catholic university faculty of biotechnology
EtBr - ethidium bromide
F - fungal inoculation
FAAS - flame atomic absorption spectroscopy
Fe - iron
$[Fe, Mn]WO_4$ - wolframite
g - gram
GROs - gentle soil remediation options
h - hours
H - shannon diversity index
HCl - hydrogen chloride
HCN - hydrogen cyanide

Hg - mercury
H_2O_2 - hydrogen peroxide
H_2SO_4 - sulfuric acid
HPO_4^{2-} - hydrogen phosphate
IAA - indole-3-acetic acid
kg - kilogram
K - potassium
min - minutes
mL - milliliter
mm - millimeter
mM - millimolar
M - molar
MDS - multidimensional scaling diagram
Mix - mixed inoculation
Mn - manganese
nm - nanometer
N- nitrogen
Na^+ - sodium ion
NaOCl - sodium hypochlorite
Ni - nickel
NH_4-Ac - ammonium acetate
NUE - nitrogen use efficiency
pmol - picomole
P - phosphorus
Pb - lead
PCR - polymerase chain reaction
PGP - plant growth promoting
PGPB - plant growth-promoting bacteria
PGPR - plant growth-promoting rhizobacteria
PSB - phosphate-solubilizing microorganisms
PUE - phosphorus use efficiency
rpm - revolutions per minute
rRNA - ribosomal ribonucleic acid
Se - selenium
SPAD - soil plant analysis development
TAE - tris-acetate-EDTA
TF - translocation factor
TSB - tryptic soy broth
µL - microliter
µm - micron

UPGMA - unweighted pair group method with arithmetic mean
v/v - volume per volume
V - vanadium
V - volts
w/w - weight per weight
W - tungsten
Zn - zinc

1. Introduction

1.1. Soil contamination

The 21st century has been marked by the increasing atmospheric CO_2 and climatic changes such as global warming, droughts and decreasing of soil organic carbon (Kumar and Verma, 2018; Laghari et al., 2016). In particular, soils can retain large amounts of carbon helping to mitigate rising in atmospheric CO_2 (Classen et al., 2015). At the same time, temperature, water deficiency, salinity and metals are major environmental stress factors related to climate change (Kumar and Verma, 2018). Indeed, metal contamination of soils, sediments, and water (Figure 1.1) has become a global concern not only due to their persistence in soils, and the related environmental hazard, but also due to health issues for both humans and animals (European Commission, 2013; Mazej et al., 2010; Pires et al., 2017).

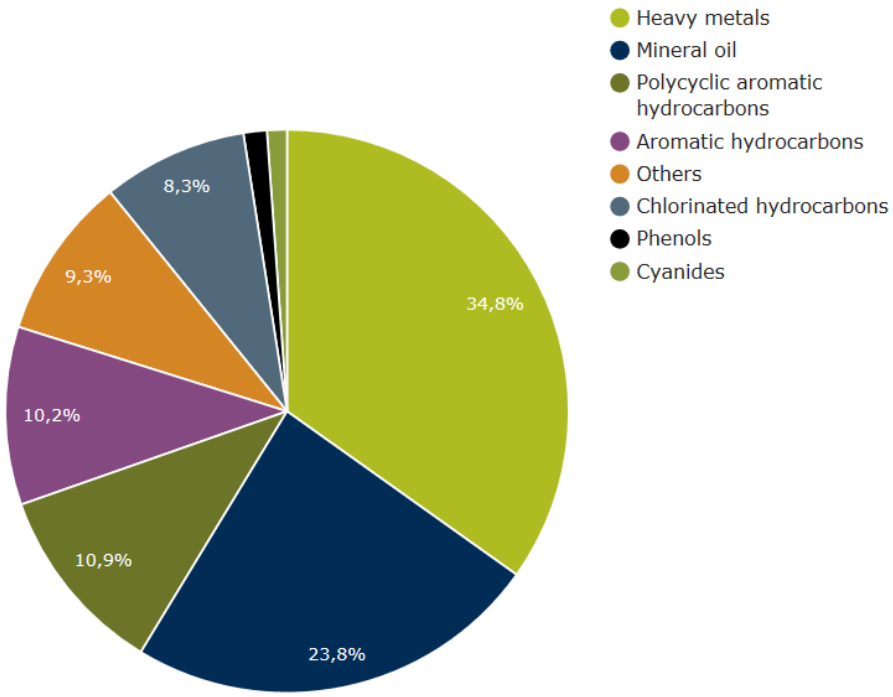


Figure 1.1 - Contaminants affecting the soil and groundwater in Europe (retrieved from EEA, 2014).

1.1.1. Anthropogenic introduction of metals in soils

Several anthropogenic activities, such as smelting, mining, use of pesticides, fertilizers and manure in agriculture, burning of fossil fuels and emissions from the industries, are responsible for accumulation of metals in soils (European Commission, 2013). Additionally, the same report estimates that in European territory, three million sites are possibly affected by these contaminants, and a quarter of a million urged for intervention or remediation.

Metals can contaminate soils and groundwater (Gall et al., 2015) and can affect human health as they can enter the human body through inhalation, ingestion (consumption of crops grown in contaminated soil, like leafy vegetables) or also by skin contact (Purakayastha and Chhonkar, 2010; Soodan et al., 2014). Consequently, metals can accumulate on body tissues of living organisms, in a process called bioaccumulation; similarly when present in food chain, metal concentration tends to increase in higher trophic levels, a process named as biomagnification (Ali et al., 2013).

The term heavy metals is generally applied for the elements that are toxic and have an atomic density higher than 6 g cm^{-3} (Pinto et al., 2016). Furthermore, these elements can be classified as essential and non-essential, according to their biological importance to organisms (Ali et al., 2013). For instance, cobalt (Co), copper (Cu), chromium (Cr) and zinc (Zn) can be categorized as biologically essential elements (Pinto et al., 2016). On the other hand, cadmium (Cd), lead (Pb) and mercury (Hg) are non-essential metals without any known biological function, being extremely toxic to humans (European Commission, 2013; Jaishankar et al., 2014; Sarwar et al., 2017). Occasionally, elements like arsenic (As) can be referred in this group, although they are recognized as metalloids (Pinto et al., 2015). With this purpose, it is crucial to recognize and understand the threats posed by contaminants and therefore promote the search for efficient and cost-effective remediation technologies to remove pollutants from the environment (Thijs et al., 2017).

1.1.2. Mining activities

Mining is one of the most ancient practices dated since the Neolithic period (Arndt et al., 2017; European Commission, 2009; Hartman and Mutmansky, 2002). In a very simple perspective, mining can be defined as the industrial activity that is responsible for the extraction of mineral substances from the Earth's crust, which have an economic and utilitarian value to humankind (Arndt et al., 2017; Hartman and Mutmansky, 2002; Jaishankar et al., 2014). Several purposes can be associated to the extracted minerals, not only in the production of electronic devices (cell phones, computers) and in means of transportation, but also in the construction sector and as fuels for energetic purposes (Arndt et al., 2017; Hartman and Mutmansky, 2002).

In the present time, consumption of minerals per capita has stabilized in developed countries (Arndt et al., 2017). However, United Nations expects that by 2050, human population will reach almost 10 billion (United Nations, 2017). As such, the global demand for mineral resources will continue to increase, driven mostly by the growing needs of the population in developing countries along with changing technologies, but also influenced by the industrial expansion with advancement of science and technology (American Geosciences Institute, 2017; Sheoran et al., 2010). For example, the demand for iron (Fe), copper (Cu), and aluminium (Al) in the forthcoming decades is expected to be fulfilled, due to the search of better habitability conditions in the emerging countries (Bloodworth and Gunn, 2008). In summary, mining industry will remain a crucial activity, since the sectors of transports and energy probably remain very reliant on these resources (Bloodworth and Gunn, 2008).

Mining exploration has several constraints, such as jurisdictional and community issues and more significantly the environmental effects (Arndt et al., 2017). Consequently, the main environmental effects are the physical alteration of the landscape, with the removing, processing and disposal of the mining wastes (tailings), and interference on soil properties such as soil pH, fertility and its microbial communities (Allan, 1997; American Geosciences Institute, 2017; Sheoran et al., 2010). Additionally, mining activity can lead to metal contamination of extensive areas (Wu et al., 2010) in the surroundings through

wind dispersion of metal contaminated particles (Lu et al., 2010) but also due to metal leaching from contaminated sites (Ávila et al., 2015).

1.2. Remediation techniques

Generally, conventional remediation techniques are based on civil engineering solutions and they can be characterized as highly destructive, very expensive, and labor intensive. These techniques deteriorate physiochemical and biological soil properties (Glick, 2010; Kidd et al., 2017; Lenoir et al., 2016; Mahar et al., 2016a). Indeed, they represent about one third of management practices characterized by excavation of tailings, followed by removal and confinement of polluted soil, which is later substituted by a non-contaminated soil (Alvarenga et al., 2018; EEA, 2014).

Gentle soil Remediation Options (GROs) have been recognized as an interesting alternative to the conventional remediation techniques, including plant-based (phytoremediation) options (Kidd et al., 2017). By definition, phytoremediation is based on the use of plants and their associated microorganisms; its efficiency can be enhanced with the application of soil amendments in order to confine or reduce total content on trace elements in soils, groundwater, surface water or sediments (ITRC, 2009; Kidd et al., 2017). The synergetic relationship between plants and their associated microorganisms can have a positive impact on plant health and growth, by increasing the uptake of nutrients but also by giving the plant better defense mechanisms against pathogens and higher tolerance to stress conditions (Kidd et al., 2017). Alkorta et al. (2004) distinguished several mechanisms used in phytoremediation (Figure 1.2):

- Phytostabilization
- Phytoextraction;
- Phytodegradation;
- Phytovolatilization;
- Phytofiltration;

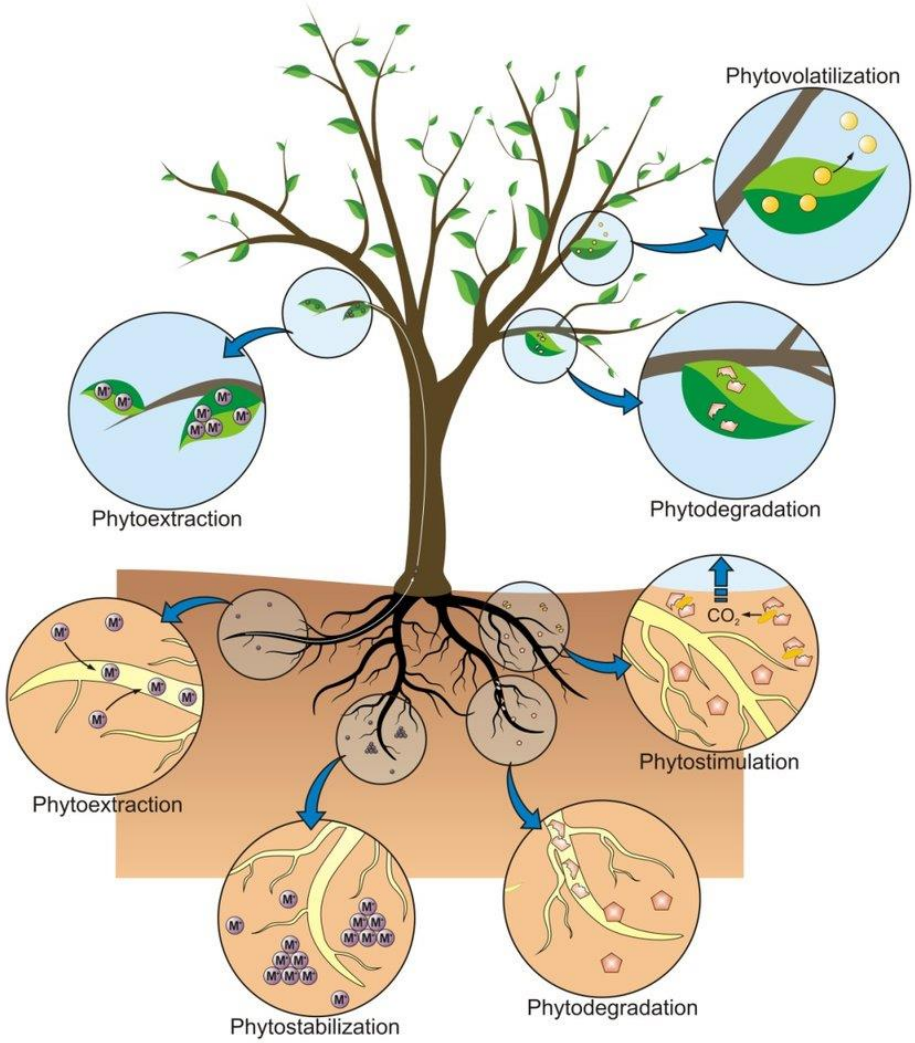


Figure 1.2 - Schematic representation of phytoremediation technologies used for both containment and removal of pollutants (retrieved from Favas et al. 2014).

Phytostabilization aims to promote the immobilization or stabilization of pollutants within the rhizosphere region (Lee, 2013; Sun et al., 2018). This immobilization process mainly intends to reduce the level of contamination in the affected area alongside with the sequestration of metal pollutants (Mahar et al., 2016a). For this situation is then desirable to use a hyperaccumulator plant that preferentially accumulates metals in their roots, thus reducing its bioavailability in the environment.

Phytoextraction is based on the uptake of contaminants by plant roots and their subsequent translocation and accumulation in aboveground tissues (Filippis, 2015;

Mehes-Smith et al., 2013). Phytoextraction may be carried out by hyperaccumulators (Lee, 2013; Ullah et al., 2015), due to their capacity to accumulate large quantities of metals in their aboveground tissues (Pinto et al., 2016). When grown in metal-enriched environments, these plants are capable to accumulate 100–1000-fold more than normal plants (Pinto et al., 2015). Hyperaccumulators are defined as plant species that are capable of accumulating metal(loid)s above the threshold concentrations of 10000 mg kg^{-1} dry weight of shoots for Zn and Mn, 1000 mg kg^{-1} for Co, Cu, Ni, As, and Se, and 100 mg kg^{-1} for Cd (Baker and Brooks, 1989; Brown et al., 1994).

In **phytodegradation**, contaminants uptake and degradation by plants, can be performed by their enzymes or through photosynthetic oxidation/reduction reactions (ITRC, 2009). In particular, rhizodegradation is based on the biodegradation of organic pollutants, carried by plant roots and microorganisms, leading possibly to its destruction (Filippis, 2015).

Phytovolatilization occurs when the pollutants are converted into volatile contaminants thus released through transpiration. This process does not require plant harvesting thus consisting as a desirable technology (Pilon-Smits, 2005).

Phytofiltration occurs in aqueous environment, where the plants are capable to absorb, concentrate and/or precipitate contaminants (e.g. metals) in their root system or in other immersed tissues (Favas et al., 2014). For this reason, their movement capacity in aqueous medium is reduced once the contaminants are absorbed by plants (Ali et al., 2013).

The phytoremediation has been documented as a cost effective method for remediation of metal contaminated soils (Sarwar et al., 2017). However, this approach also has some drawbacks, such as:

- it requires long periods of time to remove the metals (e.g. phytoextraction) (Burges et al., 2018);

- it is limited by the bioavailable fraction of contaminants for plants roots uptake (Alkorta et al., 2004; Mench et al., 2009);
- few plant species can naturally tolerate and/or accumulate high concentrations of the above mentioned environmental contaminants (Glick, 2010);
- introduction of non-native plant species in contaminated sites, thus posing a possible threat to biodiversity (Alkorta et al., 2004).

For phytoremediation purposes, the selection of plant species to be implemented must take into consideration several aspects, for instance the characteristics of the contaminated site (e.g., type of contaminant, (in)organic contaminant) along with the appropriate phytoremediation strategy to apply (Burges et al., 2018). Ideally, in order to perform an effective remediation of metal polluted soils, plants must be tolerant to one or more metals, be a highly competitive and fast growing plant, and produce a high aboveground biomass (Glick, 2010).

Helianthus annuus (sunflower), is an oil seed crop with a very substantial economic value, due to its bioenergetics traits (high oil yield) therefore being one of the most important crops worldwide (Forchetti et al., 2010; Stoikou et al., 2017). Moreover, this energy crop has also the potential for phytoremediation purposes (Marques et al., 2013). Several studies have shown sunflower as a metal accumulator (Fässler et al., 2010; Rojas-Tapias et al., 2012; Wu et al., 2006), being one of the most popular plant species used in phytoextraction (Pilon-Smits, 2005) and showing high capacity to remove contaminants from the soil (Nehnevajova et al., 2007; Stoikou et al., 2017).

With effect, combining the remediation of the contaminated soil, intending to obtain a profitable value along with benefits for the environment can be entitled as phytomanagement (Cundy et al., 2016). A comparable outlook of Phytoremediation and Phytomanagement is presented on Figure 1.3.

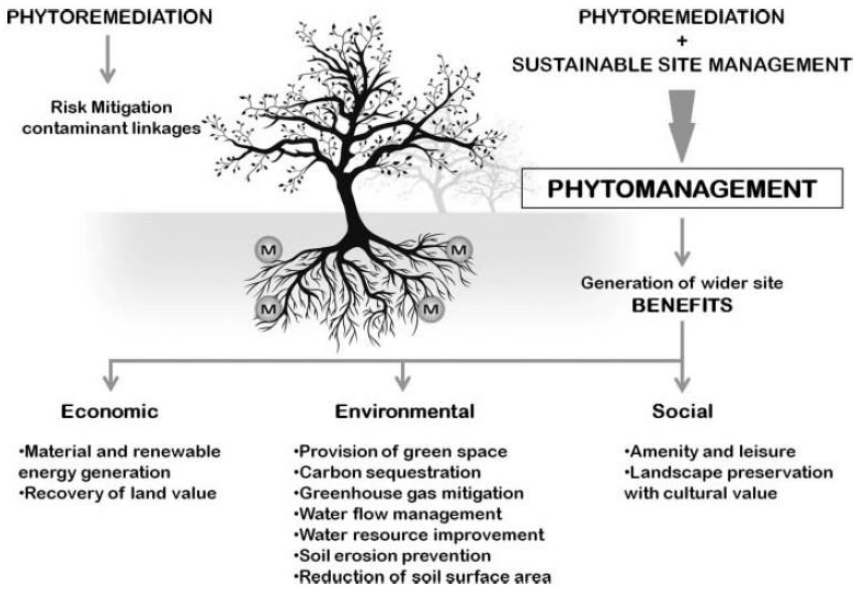


Figure 1.3 - General overview of phytomanagement approach with the respective main benefits (retrieved from Burges et al., 2018).

The transition between phytoremediation and phytomanagement takes in account the cost-benefit ratio that opens up the possibility to remediate contaminated site with reduced economic value (Burges et al., 2018).

1.2.1. Assisted-phytoremediation

In order to guarantee a more efficient remediation, phytoremediation can be assisted through the addition of microbial inoculants such as plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF), but also by the addition of inorganic and/or organic amendments in an effort to enhance plant growth (Lomaglio et al., 2018; Mishra et al., 2017). Microorganisms are able to provide nutrients for the plants but also can attenuate the harmful effects of metals on plant growth (Ullah et al., 2015).

1.2.1.1. Plant growth-promoting rhizobacteria (PGPR)

Kloepper and Schroth (1978) described PGPR as bacteria capable of colonize plants' root, aiding the plant development through direct or indirect mechanisms (Figure 1.4).

PGPR can have a direct influence in plant growth by assisting nutrient uptake from the environment, for example helping in N_2 fixation and P and K solubilization (Glick, 2012), but also inducing alterations on plants' root surface area and morphology (Bolan et al., 2011; Pérez-Montaño et al., 2014). Additionally, they can also act as regulators of phytohormones, such as auxins, cytokinis, gibberellins and ethylene, which are essential to plant growth and tissue development, as well as in plant responses to biotic and abiotic stress conditions (Gangwar et al., 2014; Glick, 2012; McNear Jr., 2013). When exposed to stress conditions, plants increase ethylene levels, thus behaving as an inhibitor of plant growth (Glick, 2010). In order to reduce plant stress, some PGPR are capable to produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which can decrease high levels of ethylene, through the consumption of its precursor (ACC) (Glick, 2014). Moreover, some PGPR produce siderophores that can be closely connected with metal uptake by plants (Ullah et al., 2015). As secondary metabolites of several microorganisms (namely bacteria and fungi), siderophores are chelating agents with high affinity for ferric ions, that are released by these organisms under iron deficiency conditions (Khan et al., 2018). Furthermore, siderophores can display affinity to other metals such as Zn, Cu, and V (Khan et al., 2018). Consequently, they can be used for metal remediation, once siderophores enhance bioavailable metal fractions in soils (Khan et al., 2018; Mench et al., 2009).

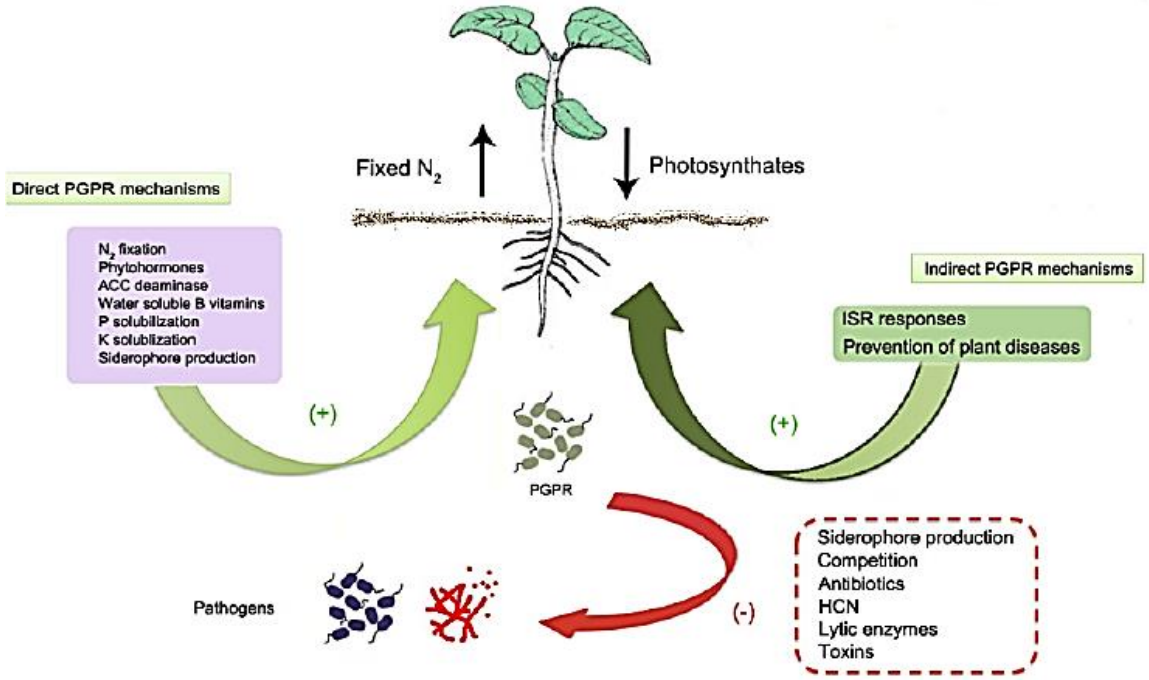


Figure 1.4 - Direct and indirect mechanisms associated with PGPR (retrieved from García-Fraile et al., 2015).

On the other hand, PGPR can also act as biocontrol agents, against phytopathogenic microorganisms through (1) the synthesis of biocides (e.g. HCN, antibiotic), (2) induction of plant systemic resistance responses, and (3) interference on bacterial Quorum Sensing (Glick, 2010; Khan et al., 2009; Pérez-Montaño et al., 2014).

Pseudomonas spp. are the most predominant group of soil microorganisms that biodegrade complex organic compounds, typically involving the concerted efforts of several different enzymes (Glick, 2010). Furthermore, they can perform as biocontrol agents, biofertilizers, but also as siderophores producers, involved in modification of metal speciation in soils (Haas and Défago, 2005; Ortíz-Castro et al., 2009; Pinto et al., 2016; Shahid et al., 2018). According to Shen et al. (2013) several *Pseudomonas* species showed plant promoting traits as nonpathogenic biocontrol agents such as *Pseudomonas chlororaphis*, *P. fluorescens and P. putida.* According to Sitaraman (2015) several *Pseudomonas* species also act as plant growth promoters' including *Pseudomonas aeruginosa* and *P. stutzeri*.

1.2.1.2. Arbuscular mycorrhizal fungi (AMF)

Microbe-assisted phytoremediation can be a promising approach for phytoremediation with an enhanced efficiency, due to the addition of AMF (Danesh et al., 2013). In a general way, AMF are able to improve plant establishment and development, by supporting water and nutrient uptake (e.g. phosphorus) from the soil but also the absorption of micronutrients like Zn and Cu (Atkinson et al., 2010; McNear Jr., 2013). On the other hand, the host plant functions as a carbon source for the AMF in this symbiotic association (Miransari, 2011).

In addition, AMF may also influence metal bioavailability in the soil and plants (Miransari, 2011). This well-known association between plant roots and soil-borne fungi might have a greater impact on the plants' tolerance to metals, since the nutrient uptake by plants is improved. Due to the expansion of mycelial network of AMF into soil volume, it significantly increases the surface area for the uptake of immobile nutrients (Crossay et al., 2017). Recently, the AMF *Rhizophagus irregularis* has been widely investigated as, a biocontrol agent (Pérez-De-Luque et al., 2017) and plant growth promoting agent (Vangelisti et al., 2018). According to Giasson et al. (2006) AMF inoculation affected the extraction of Pb, Zn and Cd of grass mixture grown in a contaminated soil. Moreover, the same AMF in association with clover was capable to accumulate Cd within its vacuoles (Yao et al., 2014). In another study, the *R. irregularis* enhanced Cu tolerance of maize Cu-sensitive cultivars (Merlos et al., 2016). Similarly, *Lotus japonicus* inoculated with *R. irregularis* showed an improvement on plant growth along with a higher resistance to Cd, perhaps explained by the P uptake (Zhang et al., 2015).

Recent works have been studying the effects of PGPR and AMF co-inoculation on plant growth and yield (Cely et al., 2016; Pereira et al., 2016; Saia et al., 2015) and on plant defense against pathogens (Pérez-De-Luque et al., 2017). Under stress conditions, this synergetic relationship can stimulate plant growth and development (Moreira et al., 2016a).

Therefore, these type of benefic associations can influence metal availability and uptake by plants (Sarwar et al., 2017) (Figure 1.5). For this reason, it is fundamental to

find the most suitable bioinoculant for the respective plant species, so that metal remediation from the soil occurs successfully (Sarwar et al., 2017).

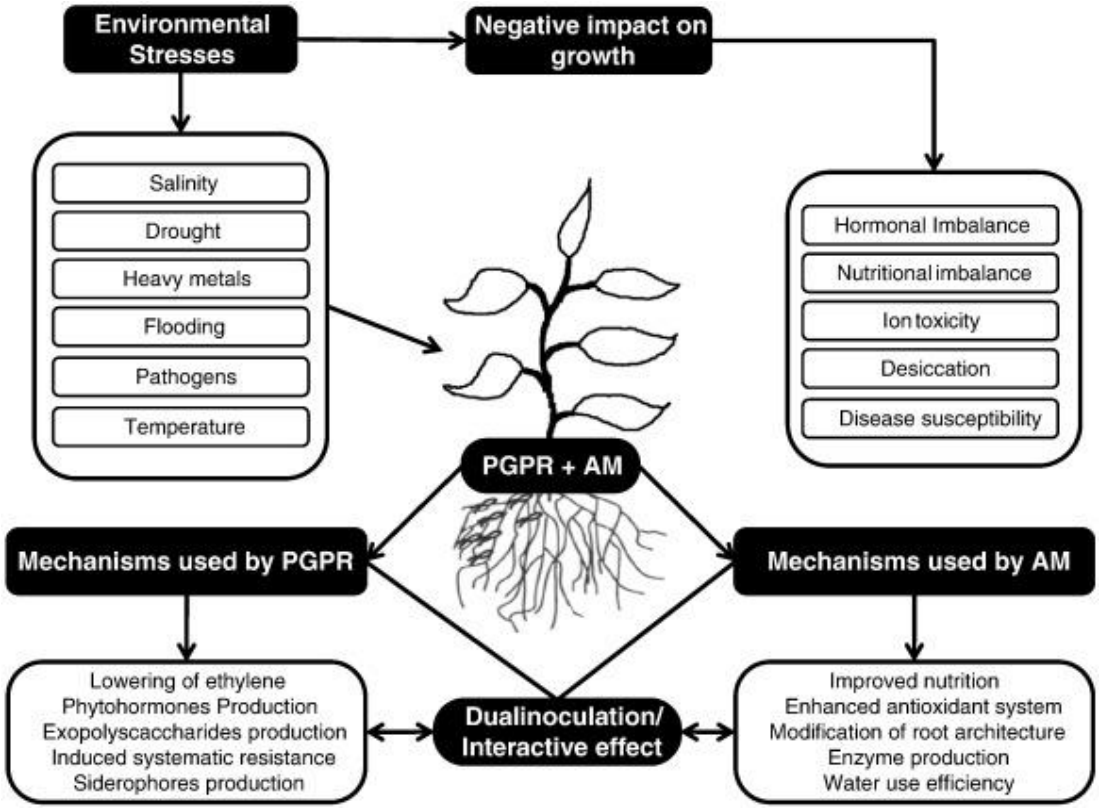


Figure 1.5 - Plant responses to biotic and abiotic stress, under microbial inoculation (with PGPR and AMF) (retrieved from Nadeem et al., 2014).

1.3. Biochar as a soil amendment

In the recent years, biochar (BC) has been widely used as soil amendment (Graber and Elad, 2013; Kang et al., 2018; Lomaglio et al., 2018; Tang et al., 2013). As a carbon-rich product, BC is obtained through the pyrolysis of organic matter (e.g. biomass) (Nartey and Zhao, 2014). Pyrolysis is a thermic decomposition of materials rich in carbon, under absence of oxygen or alternatively with low-oxygen conditions (Mandal et al., 2016; Tang et al., 2013).

The biomass feedstock and pyrolysis settings (such as temperature, residence time, pressure) affect BC's physicochemical traits including porous structure, surface area and charged surface, thus affecting BC's adsorptive capacity towards metals and organic compounds (Nartey and Zhao, 2014; Sizmur et al., 2017). Its physicochemical traits also induce changes on soil properties by increasing pH, water retention, and nutrient maintenance (e.g. prevents nitrogen leaching), which in turn may influence microbial soil community response (Tang et al., 2013; Zhu et al., 2017). Previous studies also indicate an enhanced plants' capacity for the nutrient uptake when the soil is amended with BC (Atkinson et al., 2010). BC's traits along with soil properties directly influence crops' growth and productivity (Gravel et al., 2013). Simultaneously, BC can also act as a nutrient supplier for soil microorganisms, potentially acting as regulator of nutrient cycling (Zhu et al., 2017).

Biochar has been used as an auxiliary for soil remediation in particular in phytostabilization processes (Bolan et al., 2011; Sun et al., 2018; Tang et al., 2013), as BC is capable to adsorb metals, reducing its concentration in soil and consequent bioavailability (Nartey and Zhao, 2014). As a cost-effective sorbent, BC can be associated with different metal sorption mechanisms, according to its properties and target metals (Li et al., 2017). The general metal sorption mechanisms by BC are represented in Figure 1.6.

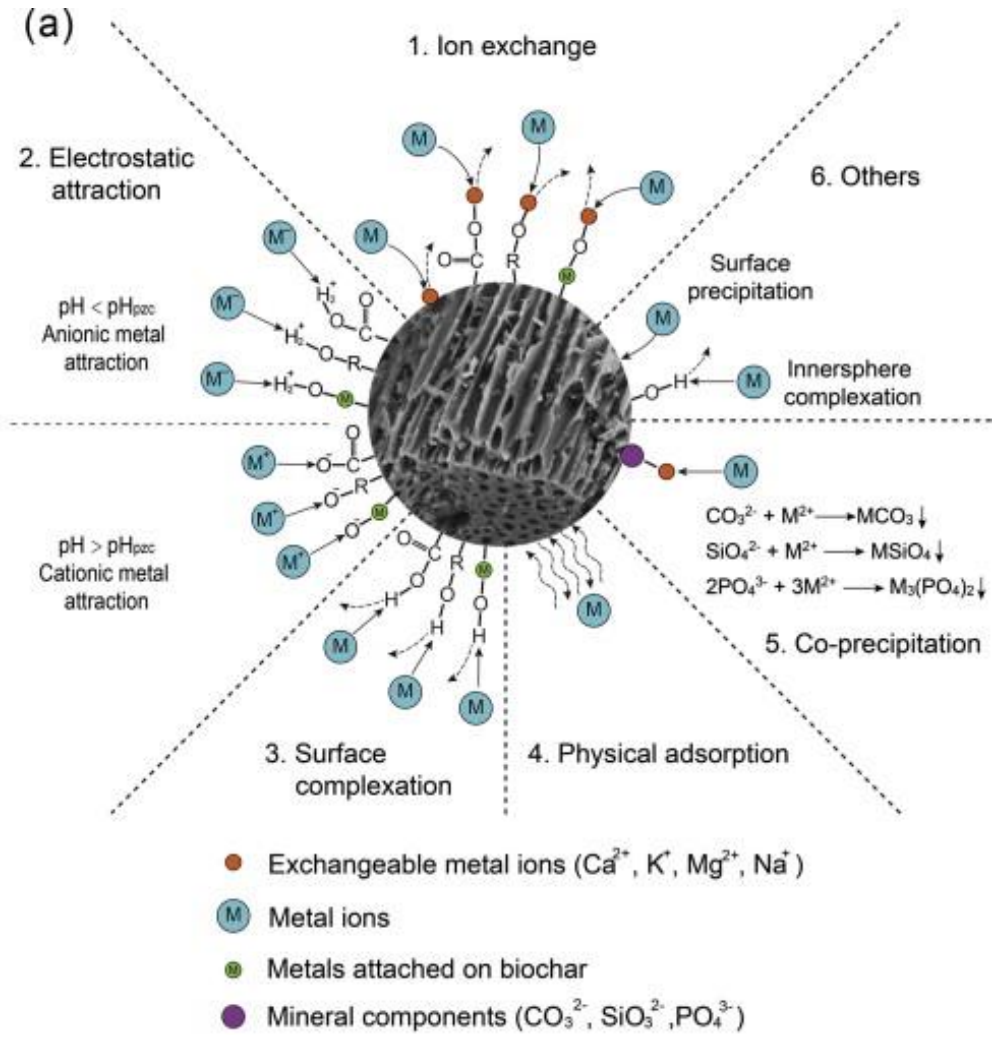


Figure 1.6 - General mechanisms proposed for BC's metal adsorption (retrieved from Tan et al., 2015).

Specific functional groups located on BC's surface (e.g. oxygen-containing groups, -OH) can establish strong connections with metals by ion exchange, electrostatic attraction and surface complexation (mechanisms 1 to 3) (Li et al., 2017; Tan et al., 2015). The ion exchange mechanism (1) is centered on the electrostatic attraction between the negative charged BC surface and anions within the soil. Electrostatic attraction (mechanism 2) occurs by both the binding between anion contaminants and a positively charged BC surface, or with the binding between cation contaminants and a negatively charged BC surface. The third mechanism represents (surface) complexation that has an important role on metal sorption, since it is based on the interaction between metal ions and functional groups (e.g. carboxylic and hydroxyl groups) present in BC surface (Li et al.,

2017). Studies demonstrated that at a lower pH, an electrostatic repulsion between BC surface and cations contaminants is registered, once they both have the same electrical charge thus lowering the adsorption rate (Tan et al., 2015). For the physical/electrostatic adsorption (4), BC surface area and its surface energy will strongly influence the adsorption of metal contaminants and further removal from the soil (Ali et al., 2017). Co-precipitation (5) generally occurs between metal cations and insoluble salts (for instance carbonate and phosphate) on the surface of BC, whose mineral content is very significant (Sizmur et al., 2017).

The mechanisms associated to BC and its influence on soil microbial growth and activities remains indistinct (Yu et al., 2016). The establishment of a cooperative relationship with soil microbiota, by giving them a more favorable soil environment, will provide a gradual nutrient release favoring the development of the plant's root (Sun et al., 2018). With effect, unveiling microbe-BC relationships could be resourceful for the association of BC traits with several soil processes such as degradation of contaminants (Zhu et al., 2017).

Salix viminalis was used to remediate a highly As and Pb contaminated site, using different BC rates and size fractions in assisted-phytoremediation (Lebrun et al., 2018). In this case, soil fertility was improved after the application of BC, regardless their particle size. This plant was not able to grow in non-amended soil, however the addition of fine BC particles reverted this situation. Overall, accumulation of metal(loid)s occurred preferentially in *S. viminalis* roots', showing its potential for phytostabilization purposes. A similar study was carried out by Sun et al. (2017) with different rates of straw BC applied on soil for maize growth. BC at 1 and 5% had a benefic effect in maize grain and straw yield.

The combination of BC and the inoculation of microorganisms can also be used as phytoremediation supporters; however, few studies have reported this association. Ali et al. (2017) showed the phytoremediation potential of the strain *Streptomyces pactum* Act12 along with BC application in a mining soil. Results showed that the bacterial strain improved metal translocation, while BC functioned as a stabilizer for trace elements in soils.

1.4. Study of the genetic diversity of soil bacterial community

Phytoremediation effectiveness is highly dependent on rhizospheric bacteria (Jiang et al., 2016). On the other hand, metals can affect diversity and abundance of these bacterial communities, since they induce a selective pressure on soil microorganisms, according to the level of metal contamination and availability at the site (Gomes et al., 2010; Sun et al., 2016; Zhang et al., 2016). In the same way, native soil microorganisms can also affect the bacterial inoculant, for instance with reduction of PGPR effectiveness (Castro-Sowinski et al., 2007).

Contaminated sites with high levels of metals constitute a source of metal tolerant microorganisms (Kidd et al., 2017) that can be used as bioinoculants in phytotechnological approaches. On the other hand, bioinoculation can incite a shift on the equilibrium of soil microbial communities (Trabelsi and Mhamdi, 2013). In an initial phase, inoculants may be able to colonize the plant although but they may not persist in the soil throughout time (Finkel et al., 2017).

In general, the most frequent methods used for plant inoculation purposes are seed and soil inoculation, with high densities of viable microorganisms that normally varies between 10^7 and 10^8 CFU mL^{-1} (Kidd et al., 2017; Trabelsi and Mhamdi, 2013). In order to have a successful inoculation, bioinoculants not only have to compete with the native microbiota in order to persist in soil, but also they have to be adapted to the variable abiotic conditions (Finkel et al., 2017).

The effect of inoculation on bacterial strains isolated from plant rhizosphere was studied by Marques et al. (2013). This study indicated that increasing concentrations of metals reduced rhizospheric bacterial diversity; however, the inoculation of PGPR attenuated this effect as bacterial diversity was maintained throughout the experience. In another study, two *Frankia*-inoculated alder species were grown in a gold mine waste rock (Callender et al., 2016). At this site, metal bioavailability was reduced through the development of these species, together with other beneficial effects, such as, enhanced abundance of certain microbial species involved in metal sequestration and degradation of contaminants.

Another study reported the effects of co-inoculation with different consortia's of PGPR and AMF on rhizosphere microbial communities as well as on wheat growth (Roesti et al., 2006). This study showed that through DGGE analysis, it was possible to recognize that combined bioinoculation (two different PGPR consortia and/or an AMF consortium) induced significant changes in native rhizosphere community. Although this effect was more evident when the PGPR consortium were applied, both treatments were able to cooperate in order to improve wheat growth.

1.5. Experimental Aims

This research thesis was part of a project devoted to the application of phytomanagement in different contaminated sites, project PhytoSUDOE. The target site was the Borralha mine, and establishment of sunflower with inoculation of PGPR and AMF were envisaged. The work of this thesis involved microcosm studies with soil collected from Borralha in which sunflower seeds were sown with and without bioinoculation and with biochar as amendment.

With this in mind, the main goals of this experiment are:

- To assess the effect of three different microbial inoculants (PGPR - *Pseudomonas reactans,* AMF - *Rhizophagus irregularis* and the combined inoculation (mixed treatment, PGPR+AMF) on biomass production and metal accumulation of sunflower plants grown in a mine soil;
- To evaluate the impact of different quantities of BC on sunflower biomass, metal availability in soil and metal accumulation in sunflower tissues;
- To evaluate the synergetic effect of BC and microbial inoculation on plant and soil parameters;
- To understand which is the more suitable bioinoculant to use, depending on the phytoremediation strategy to implement;
- To evaluate soil rhizospheric bacterial community and how it is affected by the addition of BC and by the microbial treatments applied to the soil.

2. Material and methods

2.1. Characterization of microbial inoculants

In the present work, the rhizobacteria *Pseudomonas reactans* EDP28 and the commercial AMF *Rhizophagus irregularis* were used as microbial inoculants.

The bacterial strain EDP28 was previously isolated from a metal contaminated soil located in Estarreja city (North of Portugal) and currently is part of ESB-UCP collection (Pires et al., 2017). This rhizobacteria exhibited resistance to high concentrations of Cd and Zn (Pires et al., 2017) and showed several plant growth-promoting traits (e.g. IAA and siderophore production, ACC-deaminase activity). This strain enhanced *in vitro* growth of maize seedlings exposed to increasing Zn and Cd concentrations, and it promoted metal accumulation (Cd and Zn) and nutritional status of maize plants grown in mining soils contaminated by several metal(loid)s (Moreira et al., 2016b).

The AMF *R. irregularis* was purchased to the company INOQ (Germany), consisting of 145 mycorrhizal units per cm^3 of vermiculite (1–2 mm). Previous studies showed that this AMF acted as plant growth-promoting inoculant, by increasing shoot and root biomass as well as shoot elongation of maize plants grown in a metal contaminated soil (Moreira et al., 2016a).

2.2. Soil sampling

In the present work, soil was randomly collected from Borralha mine, which is located in the city of Montalegre (district of Vila Real in the North of Portugal, Figure 2.1). Borralha has initiated its activity in 1902 and had a total yield of production 18 500 tons of Tungsten, until its closure in 1985 (Gonçalves et al., 2017; Noronha, 1983). Tungsten or Wolfram is one of the hardest metals and has the highest melting point of all metals (AMERICAN ELEMENTS, n.d.; Champion, 2012; Royal Society of Chemistry, n.d.). This metal is extracted from the mineral wolframite ([Fe, Mn]WO_4), and scheelite ($CaWO_4$)) (Barroso, 2014; Champion, 2012).

Tungsten's properties (e.g. density, hardness, melting temperature) makes this metal very required for numerous applications, for instance in production of tools with

tungsten carbide, required in the mining, oil and construction industries; moreover tungsten can be applied as a catalyst of chemical reactions (Champion, 2012; Royal Society of Chemistry, n.d.).

Figure 2.1 - Borralha mine location: Vila Real district, Portugal 41º 39’ 25.18’’ N 7º59’ 05.19’’ W.

In a long-term perspective, abandoned mines are the largest cause of environmental degradation in the mining industry (Empresa de Desenvolvimento Mineiro, 2011). Likewise, the deactivation of Borralha mine in 1986 originated since then several environmental issues such as high volumes of tailings (Figure 2.2) along with the muds and slush released from the ore exploration.

The intense mining exploitation in the Borralha area originated tailings with high concentrations of potentially hazardous trace elements but also open impoundment with rejected material (Figure 2.3) (Alvarenga et al., 2018; Ávila et al., 2015). The mechanical and chemical dispersion of these pollutants is aided by accentuated slope, runoff and infiltration of rainwater and consequently soil contamination (Ávila et al., 2015).

Figure 2.2 - Tailing of fine grain site material on Borralha mine. http://www.phytosudoe.eu/en/s3-borralha-montalegre-po/

Figure 2.3 - Open impoundment with rejected material in Borralha mine. http://www.phytosudoe.eu/en/s3-borralha-montalegre-po/

In an effort to essentially restore contaminated soils at the Southwestern Europe territory, the PhytoSUDOE project emerged. In order to restore these degraded sites, PhytoSUDOE proposes the application of phytomanagement techniques, with the cultivation of energetic crops like sunflower along, with the inoculation of

microorganisms capable to aid the soil reclamation process (e.g. soil structure, fertility and nutrient cycling) (http://www.phytosudoe.eu/en/the-project/project-summary/).

The implementation of these strategies intends to minimize the spreading of mining pollutants in an initial phase but even more to add economic value to the affected land. Several experimental plots were established in the field, which includes different plots with non-inoculated and inoculated sunflower with distinct microbial treatments (Figure 2.4).

Figure 2.4 - Established experimental plots under the scope of Phytosudoe project. http://www.phytosudoe.eu/en/phytoremediation-experiments-in-site-3-borralha-mine-portugal/.

In the present work, soil was randomly collected at this site and sieved to 2 mm. Soil properties are shown in Table 2.1.

Table 2.1 - Physicochemical properties of Borralha mine soil.

Parameter	Value	Method
pH (H_2O)	4.26 ± 0.01	Potentiometric
Texture	Silt Loam	Hydrometer
Electrical Conductivity (µS cm^1)	150 ± 2	Condutimetry
CEC ($cmol^+kg^{-1}$)	2.8 ± 0.1	
K^+ ($cmol^+ kg^{-1}$)	0.21 ± 0.04	
Mg^{2+} ($cmol^+ kg^{-1}$)	0.26 ± 0.00	
Ca^{2+} ($cmol^+ kg^{-1}$)	1.6 ± 0.0	
Na^+ ($cmol^+ kg^{-1}$)	0.07 ± 0.00	
Phosphorus (P_2O_5) (mg kg^{-1})	437.0 ± 0.4	Mehlich 3
Potassium (K_2O) (mg kg^{-1})	129.6 ± 1.3	Mehlich 3
Calcium (CaO) (mg kg^{-1})	540.9 ± 0.9	Mehlich 3
Magnesium (MgO) (mg kg^{-1})	64.2 ± 0.4	Mehlich 3
Total N (%)	0.31 ± 0.02	Condutimetry
Organic C (%)	4.13 ± 0.04	Condutimetry
Total metal(loid)s (mg kg^{-1})		
Co	20.49 ± 0.41	
Mn	638.93 ± 12.78	
Fe	14523 ±290.5	
V	21.19 ± 0.42	
As	34.98 ± 0.70	
Cd	1.45 ± 0.05	*Aqua regia*
Cu	1080.0 ± 0.50	*Aqua regia*
Ni	13.49 ± 0.15	*Aqua regia*
Pb	71.06 ± 0.05	*Aqua regia*
Zn	228.24 ± 0.70	*Aqua regia*
Hg (µg kg^{-1})	<600	*Aqua regia*

2.3. Pot experiment

The experiment consisted of a factorial design with four microbial treatments (C, B, F and Mix) and four levels of BC (0, 2.5, 5, and 10 % (w/w)), as follows:

- non-inoculated soil with sunflower (control - C);
- inoculated soil with the bacteria *P. reactans* and sunflower (B);
- inoculated soil with an AMF commercial and sunflower (F);
- inoculated soil with a mixture of *P. reactans* and AMF and sunflower (Mix).

Each treatment was replicated five times, consisting on a particular combination of mining soil, BC, and microbial inoculants (Figure 2.5). The BC used has a particle size ≤6 mm and was purchased to Ibero Massa Florestal, S.A., Portugal.

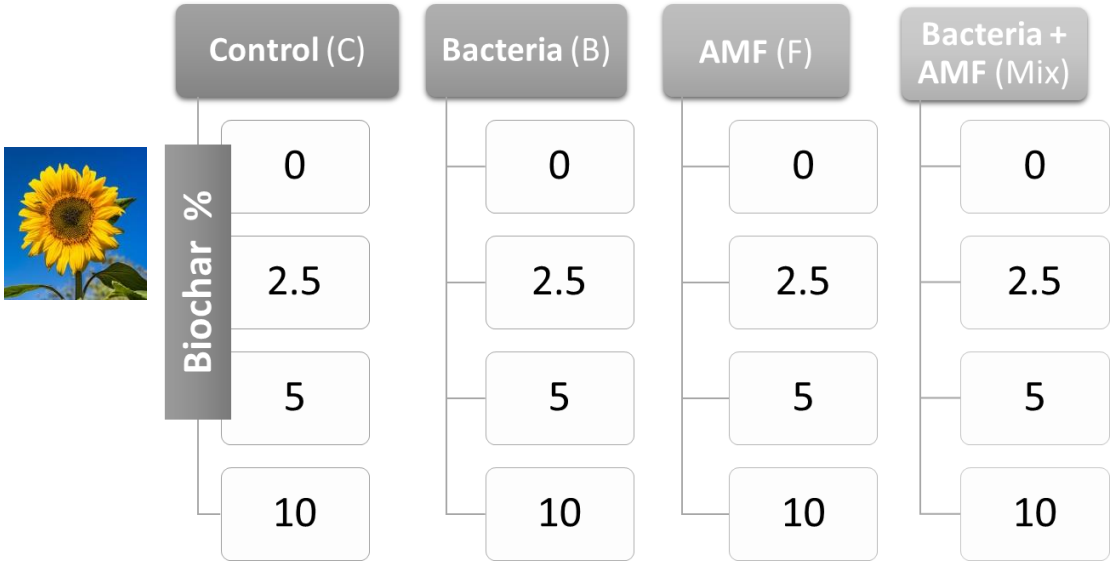


Figure 2.5 - General representation of the four microbial treatments applied to the mine contaminated soil and percentages of BC added.

Sunflower seeds were sterilized using a solution of NaOCl (50% v/v) during 10 min and then rinsed with deionized sterilized water, until the resultant water came out colorless. Then, 10 seedlings were sowed and disposed in a water-agar media for 7 days. Each pot containing 1 kg of soil or soil amended with BC received six sunflower seedlings that were afterwards thinned to five.

The AMF *R. irregularis* was mixed in the soil according to the recommendations of manufacture (100 mL kg $^{-1}$ soil) five days before the sowing.

The bacterial strain EDP28 was grown in Tryptic Soy Broth (TSB) media overnight at 100 rpm and 30^{o} C. Then, 25 mL of the bacterial inoculum (10 ^{8} CFU mL^{-1}) were sprayed in soil of B and Mix treatments. With the same intent, 25 mL of a diluted and non-inoculated TSB solution (1:1 sterilized water/TSB) were applied in the pots corresponding to C and F treatments. Two inoculations were performed, one and four weeks after the sowing, respectively.

Pots were watered 3 times per week, with an average volume of 80 mL of tap water. Once a week the pots were rotated in the same direction, in order to have an equal distribution of radiation. The plants were maintained under a controlled environment in a growth room (12 h of photoperiod, temperature 20-24^{o}C) since the beginning of the experiment. The harvest occurred after 12 weeks.

2.4. Plant analysis

The SPAD-502 meter (Konica Minolta) is a portable device used for the rapid and non-destructive measurement of chlorophyll leaf amounts. SPAD values can also function as an indicator for leaf nitrogen amount or plant health (Ling et al., 2011). Two days before harvest, three reads were taken on the second fully expanded leaf of each plant (counting from the plant top, on opposite leaves).

After 12 weeks, plants were harvested and divided in shoots and roots. Shoot elongation and fresh biomass were determined. Then roots were carefully washed with tap water, HCl 0.1 M solution, and deionized water to remove both soil and BC particles. Plant tissues were dried at 60^{o} C, during one week to determine shoot and root dry biomass. Samples were then grinded (Culatti, Micro Impact Mill) and 0.150 g was used for acid digestion by adding an equal volume (4.5 mL) of sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2). The digestion of the samples was performed on a Berghof Speed Wave MWS-3 + Microwave Digestion System, with a rotor for 12 samples according to the following program displayed on Table 2.2:

Table 2.2 - Experimental conditions used in the Digestion system for sample digestion.

	1	2	3	4	5
Temperature (°C)	160	190	190	100	100
	21	41	41	21	20
	5	10	10	2	2
	5	5	5	1	1
	40	80	80	20	20

2.4.1. Metal analysis

The amount of metals (present at higher concentrations in soil - Cd, Cu, Pb, and Zn) accumulated in plant tissues was determined by Flame Atomic Absorption Spectroscopy (FAAS) using a Unicam-969 AA Spectrometer. All the digests were filtered through a 0.45-µm cellulose acetate filter (Sartorius) before analysis.

The digested samples showed residual Pb concentrations. Cd levels were not determined due to the malfunctioning of the Spectrometer's lamp. This analysis will be done as soon as possible.

Root and shoot bioconcentration factor (BCF) and the translocation factor (TF) were determined for Zn and Cd according to Ali et al. (2013) as follows:

$$\text{Bioconcentration factor (BCF)} = \frac{C_{\text{harvested tissue}}}{C_{\text{soil}}}$$

where $C_{\text{harvested tissue}}$ is the metal concentration in plant harvested tissue and C_{soil} is the concentration of the same metal in the soil.

$$\text{Translocation factor (TF)} = \frac{C_{\text{shoot}}}{C_{\text{root}}}$$

where C_{shoot} is the metal concentration in plant shoots and C_{root} is the metal concentration in plant roots.

2.4.2 Nutrient content

The content of N and P in plant tissues were determined using colorimetric methods according to Walinga et al. (1989). The reagents and solutions used are shown in Table 2.3.

Table 2.3 - Reagents and solutions used for the determination of N and P.

N determination	P determination
Solution 1: ▪ 50 mL of 1 M sodium salicylate ▪ 100 mL of 1mM sodium nitroprusside ▪ 5 mL of 3 mM EDTA ***Solution 2:*** ▪ 200 mL of 50 mM disodium hydrogen phosphate buffer (pH 12.3) ▪ 50 mL of 4% hypochlorite	***Solution 1:*** 80 mL of the following mixture, diluted on 300 mL of water: ▪ 15 mL of 5 mM ammonium molybdate ▪ 50 mL of 2.5 M sulfuric acid ▪ 30 mL of 30 mM ascorbic acid ▪ 5 mL of 6 mM antimonyl tartrate

Nutrient use efficiency in sunflower plants was calculated for N and P, according to Baligar et al. (2001) as follows:

$$\text{Nutrient Use Efficiency} = \frac{Total\ plant\ dry\ weight}{Total\ nutrient\ absorbed}\ \frac{g/plant}{g/plant}$$

where total nutrient absorbed is calculated as follows:

$$\text{Total nutrient absorbed} = \frac{Total\ Biomass}{Nutrient\ Concentration}$$

2.5. Soil analysis

Rhizospheric soil was taken from each pot at harvest for the determination of metal bioavailability. According to De Koe (1994), Milli-Q water and ammonium acetate (NH_4- Ac) - extractable metal fractions were obtained mixing 1 g of soil with 5 mL of water and 1 M NH_4–Ac, respectively. The suspensions were incubated at 30^oC and 150 rpm for 2 h, and then centrifuged and filtered through a 0.45 µm cellulose acetate filter (Sartorius). The Cu and Zn extractable forms were determined by FAAS. Pb was not detected and Cd levels were not assessed due to the malfunctioning of the lamp.

2.5.1. Soil bacterial community

In order to analyze the soil bacterial community, a composed rhizospheric soil sample from the five replicates for each treatment was taken in the closest proximity to plant root, and kept at -20^oC until analysis. Rhizospheric soil samples were taken in three different time points for this experiment: $t_{0,}$ sampling of the initial mining soil; $t_{1,}$ sampling 18 days after the first inoculation; $t_{2,}$ sampling 13 days after the second inoculation and $t_{f,}$ sampling at the plant's harvest.

Total DNA extraction was performed with the Power Soil DNA Isolation Kit (MO BIO Laboratories, Inc., USA) following manufacturer's procedures. The extracted DNA was maintained at -20^oC until the DGGE procedure.

2.5.2. 16S rRNA gene polymerase chain reaction (PCR)

The initial amplification of 16S rRNA gene was carried out using the universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGYTACCTTGTTAACGACTT) (Lane, 1991). The final volume for the reaction mixture was 25 µL divided into 16.25 µL of sterilized deionized water, 6.25 µL of NZYTaq II 2x Green Master Mix (NZYtech), 7.5 pmol of each primer and 1 µL of the target DNA. All the PCR reactions were performed in a Bio-Rad MyCycler Thermal Cycler (Bio-Rad Laboratories) using the following program: initial

denaturation at 94ºC for 3 min followed by 30 cycles of 1 min at 94ºC, 1 min at 52ºC and 1 min at 72ºC. A final extension step at 72ºC was performed for 10 min.

The second round for PCR reaction (Nested PCR) used the primers 338F-GC (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG_ACTCCTACGGGAGGCAGCAG) and 518R (ATTACCGCGGCTGCTGG), for the amplification of the highly variable V3 region of bacterial 16S rRNA gene (Muyzer et al., 1993). The final volume for this reaction mixture was 25 µL divided into 16.25 µL of sterilized water, 6.25 µL of NZYTaq II 2x Green Master Mix (NZYtech), 7.5 pmol of each primer and 1 µL of product PCR from the first reaction. For this PCR reaction, it was used the following program: the initial denaturation occurred at 94ºC for 5 min followed by 30 cycles of denaturation (30 sec at 92ºC), annealing (30 sec at 55ºC) and extension (30 sec at 72ºC). A final extension step at 72ºC was performed for 30 min. Fragment's amplification was verified on 1.5% agarose gel in TAE 1x (Tris-acetate-EDTA) and then stained with Ethidium Bromide for 20 min.

2.5.3. DGGE

DGGE was performed on a D-Code Universal Mutation Detection System (Bio-Rad). The PCR products resultant of the Nested PCR containing approximately were loaded in 8% polyacrylamide gel (37.5:1 acrylamide bisacrylamide) in 1x TAE buffer using a gradient ranging from 35 to 62.5% of urea and formamide (100% denaturant contains 7 mol L^{-1} urea and 40% formamide). Stage 1 of electrophoresis had the duration of 15 min and was performed at 20 V, while stage 2 lasted 960 min at 70 V. Gels were stained with EtBr during 10 min. The revelation of the gels was made with Gel Doc™ XR+ Gel Documentation System (Bio-Rad Laboratories). Both gels contained a standard of eight bands, in order to obtain a gradient of bands and as a term of comparison for the samples. Each band represents a different bacterial clone from gene libraries previously described in Henriques et al. (2004) (Band 1 - clone RAI-70; Band 2 - clone RAN-60; Band 3 - clone RAI-3; Band 4 - clone RAI-43; Band 5 - clone RAN-18; Band 6 - clone RAN-12; Band 7 - RAN-140; Band 8 - clone RAI-76).

DGGE banding profiles at the beginning of the experiment (t_0), after the first (t_1) and second (t_2) inoculation, and at the end of the experiment (t_f) were analyzed using Bionumerics software (version 6.6, Applied Maths, St.-Martens-Laten, Belgium). DGGE profiles were compared using Jaccard's similarity coefficient with 1 % tolerance and clustered according to the UPGMA method, using band-based criteria (presence and absence of the bands in the DGGE profiles). An abundance matrix was used for assembling a multidimensional scaling diagram (MDS), a two-dimensional diagram where each DGGE samples are positioned as a single point so that related samples are assembled together (Moura et al., 2009). MDS analysis was performed with Primer 6 software package (Clarke and Gorley, 2006).

DGGE patterns were also analyzed using three indexes, the Shannon-Weaver index of diversity, *H* (Shannon and Weaver, 1963), the equitability index, *E* (Pielou, 1975) and Simpson index of diversity, D (Simpson, 1949), calculated for each sample as follows:

$$H = -\Sigma \left(\eta i / N\right) \log\left(\eta i / N\right)$$

$$E = H / \log S$$

$$D = 1 - \Sigma \left(\eta i / N\right)^2$$

where ηi is the peak intensity of each DGGE band, N is the sum of the surfaces for all peaks in a certain sample and S is the number of DGGE bands found on each sample (Fromin et al., 2002).

2.6. Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics program (IBM, New York, USA, Version 24.0). A two-way ANOVA was performed for each dependent variable (as enumerated below) versus the independent variables, BC percentage (B) and microbial inoculation treatment (I).

- shoot elongation and shoot fresh and dry biomass;
- root dry biomass;
- SPAD values on plant leaves;
- nutrient content, e.g. nitrogen and phosphorus, on sunflower shoots and roots;
- nutrient use efficiency for N and P;
- metal determination, e.g. copper and zinc, on sunflower shoots and roots;
- bioconcentration and translocation factor for Cu and Zn;
- extractable Cu and Zn soil fractions.

One-way ANOVA was also performed for each BC percentage to assess the effects of each inoculation treatment on the same parameters described above. The Duncan post hoc test was performed to determine the significant ($P<0.05$) statistical differences between inoculation treatments.

3. Results

3.1. Plant analysis

3.1.1. Shoot elongation and fresh biomass

Shoot elongation values measured at sunflowers' harvest stage are presented in Figure 3.1 A, while shoot fresh biomass values are displayed in Figure 3.1 B.

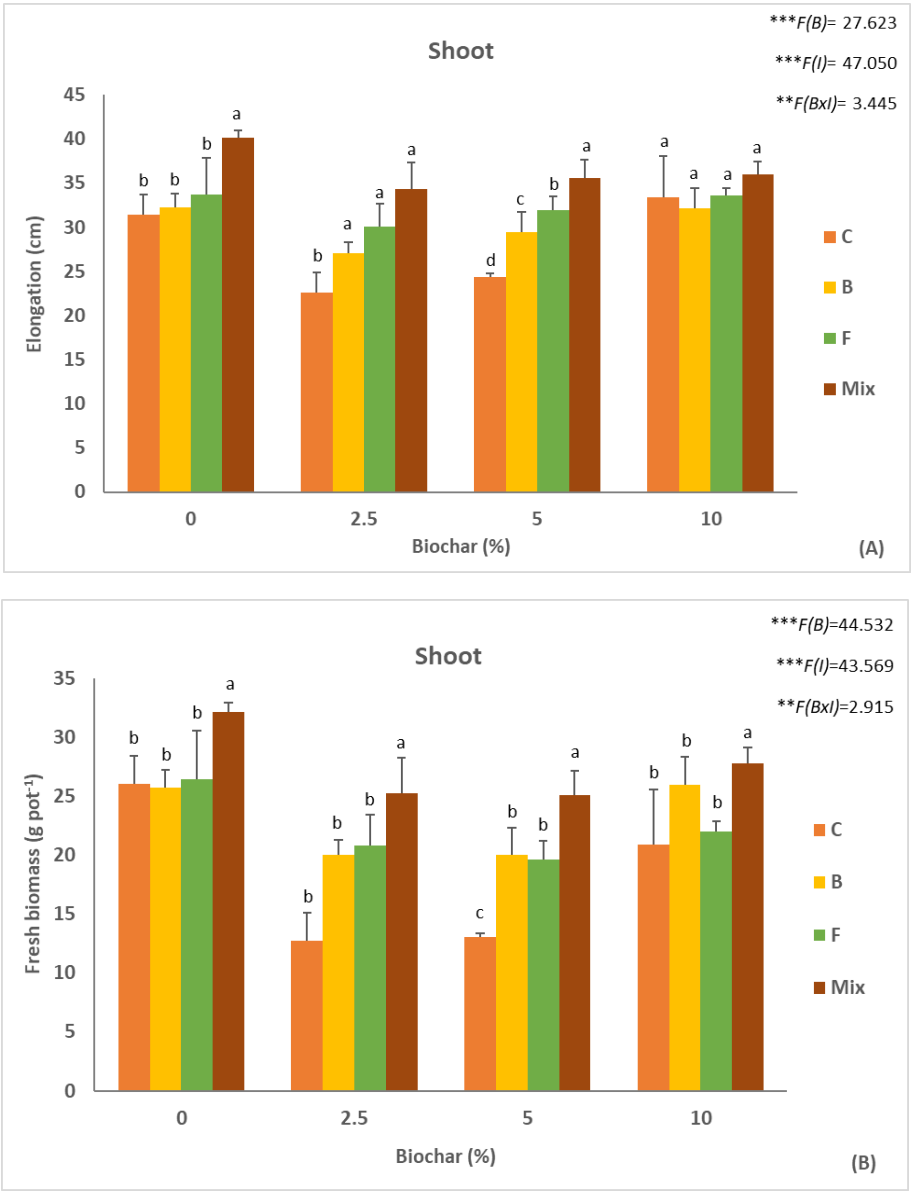


Figure 3.1 - Shoot elongation (A) and shoot fresh biomass (B) of sunflower plants grown in a mining soil amended with different BC percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants (C- non-inoculated; B - rhizobacteria *P. reactans*; F - AMF *R. irregularis*; Mix - mixture of B/F). Results are expressed as the mean value ± SD (n=5). A two-way ANOVA was

performed to determine the influence of biochar and inoculation treatment, in shoot elongation and fresh biomass. The results are shown with the test statistic for each case (B—biochar level; I—inoculation treatment; BxI—biochar x inoculation treatment interaction). A one-way ANOVA was performed to determine the influence of inoculation treatment (C, B, F, Mix) on shoot elongation and shoot fresh biomass for each biochar percentage. Means for inoculation treatments with different letters are significantly different from each other (P<0.05) given by the Duncan test. For elongation, the values of one-way ANOVA are ***F=12.437, ***F=21.781, ***F=36.473 and NSF=1.745, and for shoot fresh biomass *F=4.135, ***F=20.024, ***F=26.300 and ***F=10.467, respectively for 0, 2.5, 5 and 10% of BC. The results are displayed as *NS*- Non-significant at the level P>0.05; *significant at the level P<0.05; **significant at the level P<0.01; ***significant at the level P<0.001.

Elongation values ranged from 22.59 to 40.20 cm. According to the results of the two-way ANOVA, shoot elongation was significantly (P<0.05) influenced by BC doses and by microbial inoculants. Plants grown at 2.5 and 5% of BC showed reductions in shoot elongation in average of 28 and 22%, respectively, when compared to the control (0% BC) (Figure 3.2).

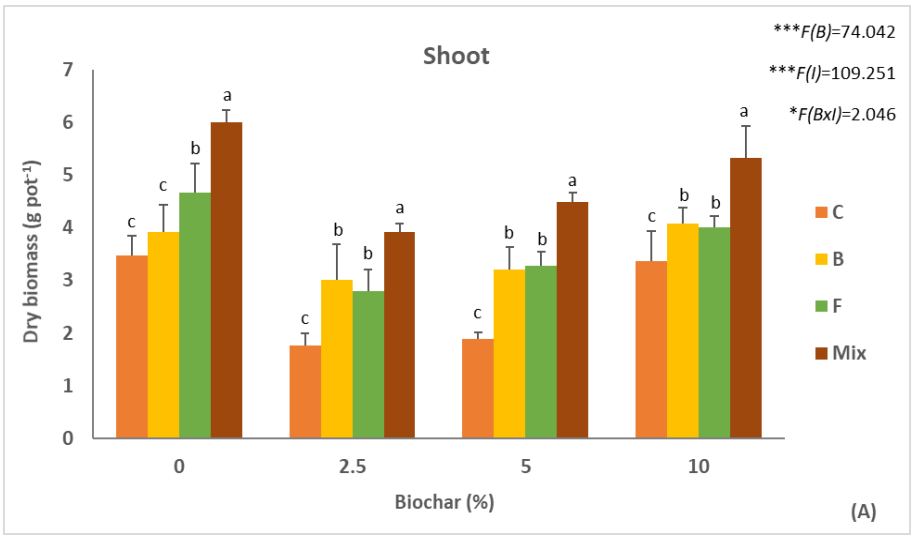
Figure 3.2 - Experimental pots at the harvest stage of sunflower plants grown in a mining soil amended with different BC percentages: **A -** no addition of BC; **B, C** and **D** - addition of BC at 2.5, 5 and 10% respectively; Each pot was also treated with different microbial inoculants represented by the letters C- non-inoculated; B - rhizobacteria *P. reactans*; F - AMF *R. irregularis*; Mix - mixture of B/F).

In general, microbial inoculants increased significantly this parameter both in the absence and presence of BC, with exception of plants grown at 10% of BC where inoculants did not induce significative (P>0.05) differences. The positive effect of microbial inoculation was particularly observed at 2.5 and 5% of BC. In general, Mix inoculation showed the best performance followed by F treatment.

A similar trend was observed for shoot fresh biomass, the values varied between 12.78 and 32.21 g, and both BC addition and microbial inoculation had a significative (P< 0.05) effect on biomass values (Figure 3.1 B). In general, BC decreased plant biomass, with plants grown at 2.5 and 5% of BC showing reductions in average of 51 and 49%, respectively in comparison to the plants grown in the absence of this amendment. However, microbial inoculants, especially Mix treatment, significantly (P<0.05) increased shoot fresh biomass of plants grown at both BC percentages, as well as in the absence of BC.

3.1.2. Shoot and root dry biomass

The values for shoot and root dry biomass are respectively presented in Figures 3.3 A and 3.3 B.



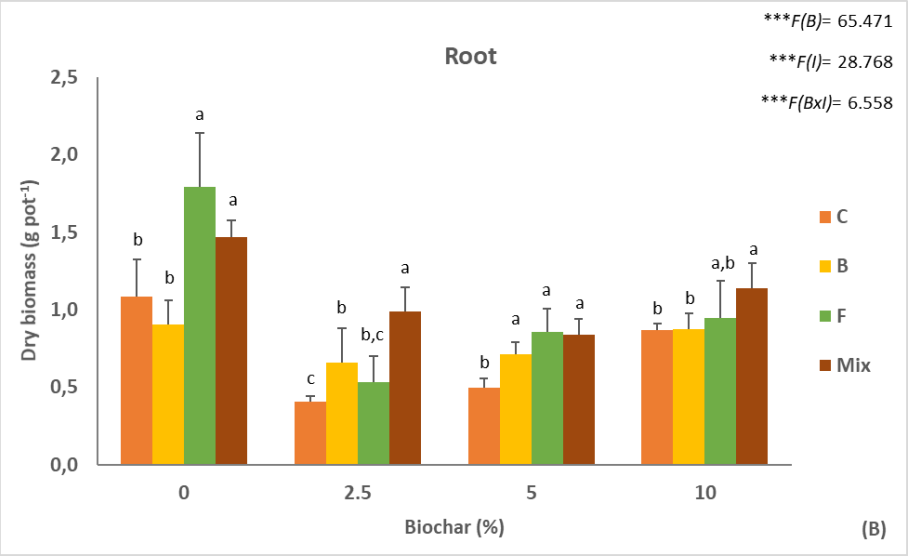


Figure 3.3 - Shoot (A) and root (B) dry biomass of sunflower plants grown in a mining soil amended with different BC percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants (C- non-inoculated; B - rhizobacteria *P. reactans*; F - AMF *R. irregularis*; Mix - mixture of B/F). Results are expressed as the mean value ± SD (n=5). A two-way ANOVA was performed to determine the influence of biochar and inoculation treatment, in shoot and root dry biomass. The results are shown with the test statistic for each case (B—biochar level; I—inoculation treatment; BxI—biochar x inoculation treatment interaction). A one-way ANOVA was performed to determine the influence of inoculation treatment (C, B, F, Mix) on shoot and root dry biomass for each BC percentage. Means for inoculation treatments with different letters are significantly different from each other (P<0.05) given by the Duncan test. For shoot dry biomass, the values of one-way ANOVA are ***F=31.148, *** F=22.658, ***F=68.829 and ***F=16.277, and for root dry biomass ***F=14.252, *** F=12.621, ***F=17.679 and *F=3.435, respectively for 0, 2.5, 5 and 10% of BC. The results are displayed as *NS* - Non-significant at the level P>0.05; *significant at the level P<0.05; **significant at the level P<0.01; ***significant at the level P<0.001.

Shoot dry biomass ranged from 1.578 to 5.999 g (Figure 3.3 A) while root biomass varied between 0.406 and 1.791 g (Figure 3.3 B). According to the results of the two-way ANOVA, both parameters were significantly (P<0.05) influenced by BC and by microbial inoculation. At 2.5 and 5% of BC it was observed a decrease of 49 and 46%, in shoot biomass, respectively in comparison to the plants grown without BC. Similar results were observed for roots (Figure 3.3 B) where it was observed a decrease of 62, 54 and 19 %, at 2.5, 5 and 10% of BC, respectively. These reductions were attenuated by bioinoculation in both plant organs, since microorganisms increased significantly (P<0.01) both parameters. Overall, Mix inoculation and F were the better treatments, even in the absence of BC.

3.1.3. Leaves chlorophyll relative content (SPAD)

This analysis was performed in order to assess if chlorophyll levels variation recorded on sunflower leaves' was related to plant nutritional status in the harvest stage. The SPAD values varied between 30.34 and 34.96 (Table 3.1). According to the results of the two-way ANOVA, SPAD values were significantly ($P<0.05$) influenced by BC percentage and by inoculation treatment. The addition of 2.5 and 5% BC induced an overall decreasing in SPAD values. In opposition, SPAD values were generally higher at 0 and 10% BC. Overall, inoculation influenced positively ($P<0.01$) this parameter. The treatments that better performed were the Mix and B treatments. However, the plants grown in soils amended with 10% of BC showed high SPAD values only when rhizobacteria (B) was applied.

Table 3.1 - SPAD values measured on leaves of sunflower plants grown in a mining soil amended with different BC percentages (0, 2.5, 5, and 10%) and treated with different microbial inoculants at the end of the experiment (harvest).

Inocula	Biochar %			
	0	**2.5**	**5**	**10**
C	31.78 ± 1.09^{c}	30.34 ± 1.24^{b}	31.91 ± 0.79^{c}	33.27 ± 0.61^{b}
B	34.30 ± 1.07^{b}	31.31 ± 1.67^{b}	33.37 ± 0.95^{a,b}	34.67 ± 1.18^{a}
F	34.96 ± 0.35^{a,b}	31.50 ± 0.93^{b}	32.20 ± 0.82^{b,c}	33.31 ± 1.14^{b}
Mix	36.14 ± 1.45^{a}	33.94 ± 1.60^{a}	34.39 ± 1.16^{a}	33.15 ± 0.76^{b}
	***F=14.883	**F=6.055	**F=7.319	NSF=2.835
	*****F(B)= 18.881**			
	*****F(I)= 18.771**			
	*****F(BxI)= 4.074**			

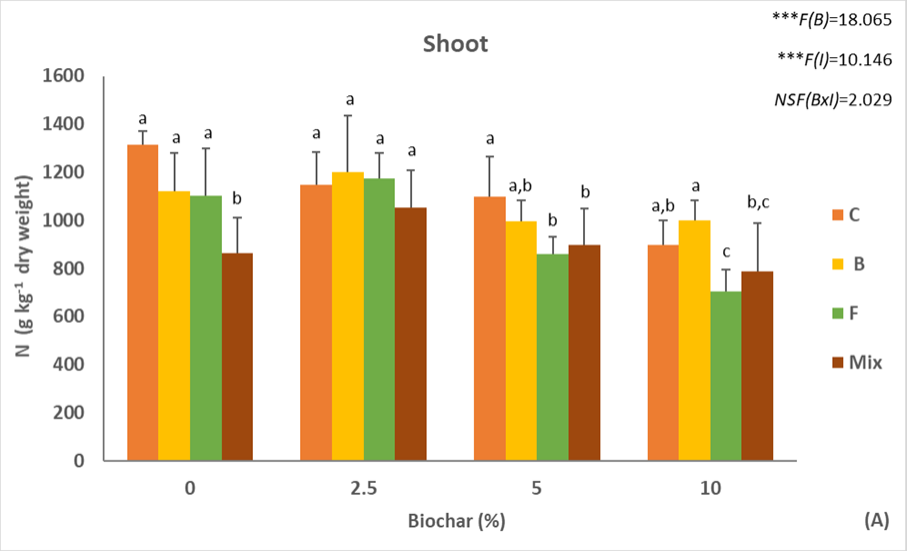
Results are expressed as the mean value ± SD (n=5). A two-way ANOVA was performed to determine the influence of BC and inoculation treatment, in SPAD values. The results are shown with the test statistic for each case (B—biochar level; I—inoculation treatment; BxI—biochar x inoculation treatment interaction). A one-way ANOVA was performed to determine the influence

of inoculation treatment (C- non-inoculated; B - bacteria *P. reactans;* F - AMF *R. irregularis;* Mix - mixture of B/F) on SPAD values variation for each BC percentage. Means for inoculation treatments with different letters are significantly different from each other ($P<0.05$) given by the Duncan test. The results are displayed as *NS*- Non-significant at the level $P>0.05$; *significant at the level $P<0.05$; **significant at the level $P<0.01$; ***significant at the level $P<0.001$.

3.1.4. Nutrient content in plant tissues

3.1.4.1. Nitrogen (N)

In general, N accumulation was higher in shoots (Figure 3.4 A) than in roots (Figure 3.4 B). N accumulation in both plant tissues was significantly (P<0.05) influenced by BC percentage. Indeed, increasing BC doses induced a decrease of 13, 16 and 32% in N accumulation in shoots, at 2.5, 5 and 10% of BC, respectively, while in roots it was observed a slight decrease in N accumulation of plants grown at 10% of BC. Overall, microbial inoculation did not influence N accumulation in both tissues.



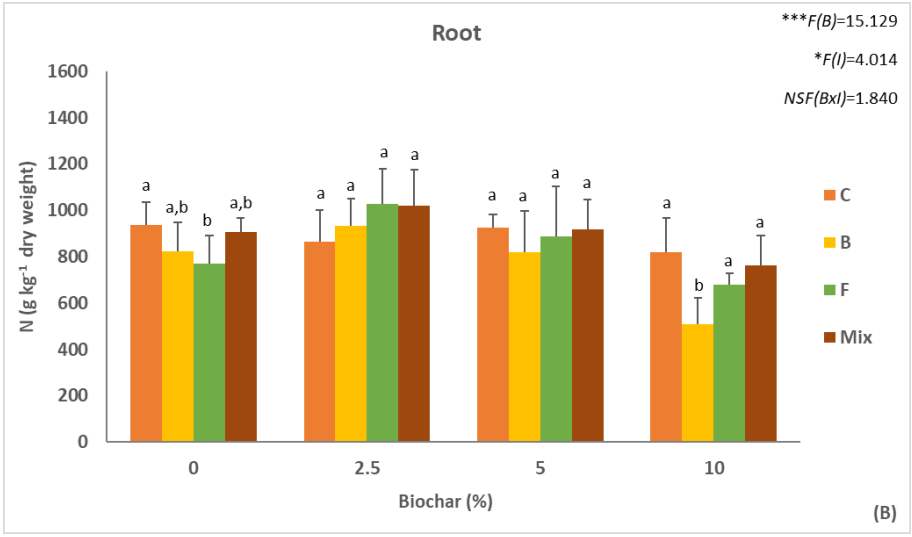


Figure 3.4 - Shoot (A) and root (B) nitrogen content of sunflower plants grown in a mining soil amended with different BC percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants (C- non-inoculated; B - rhizobacteria *P. reactans*; F - AMF *R. irregularis*; Mix - mixture of B/F). Results are expressed as the mean value ± SD (n=5). A two-way ANOVA was performed to determine the influence of biochar and inoculation treatment, in shoot and root N content. The results are shown with the test statistic for each case (B—biochar level; I—inoculation treatment; BxI—biochar x inoculation treatment interaction). A one-way ANOVA was performed to determine the influence of inoculation treatment (C, B, F, Mix) on shoot and root N content for each BC percentage. Means for inoculation treatments with different letters are significantly different from each other (P<0.05) given by the Duncan test. For shoot N content, the values of one-way ANOVA are **F=6.401, NSF=0.881, *F=3.265 and *F=4.923, and for root N content NSF=2.631, NSF=1.521, NSF=0.458 and **F=6.990, respectively for 0, 2.5, 5 and 10% of BC. The results are displayed as *NS*- Non-significant at the level P>0.05; *significant at the level P<0.05; **significant at the level P<0.01; ***significant at the level P<0.001.

Nitrogen use efficiency (Table 3.2) was significantly (P<0.05) influenced by BC addition and by inoculation. In a general way, NUE increased with the addition of BC, especially at 5 and 10% BC, with an average increment of 17 and 28% respectively, when in comparison to the plants grown in non-amended soil. Bioinoculation increased NUE by 32 and 18% in plants grown at 0 and 10% of BC, respectively when compared to the non-inoculated plants at the same BC percentages. The Mix inoculation was the treatment that better performed especially at 10% of BC.

Table 3.2 - Nitrogen Use Efficiency (NUE) in sunflower plants grown in a mining soil amended with different BC percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants at the end of the experiment.

Inocula	Biochar %			
	0	**2.5**	**5**	**10**
C	831.33±40.47^{c}	918.58±67.45^{a}	999.90±58.62^{b}	1 148.23±136.35^{b}
B	948.98±124.17^{b}	926.80±54.83^{a}	1 046.87±99.10^{a,b}	1 104.60±95.55^{b}
F	920.27±35.18^{b,c}	868.67±53.07^{a}	1 106.21±34.23^{a}	1 442.44±133.43^{a}
Mix	1 223.32±34.04^{a}	961.63±92.76^{a}	1 044.85±43.37^{a,b}	1 403.26±171.75^{a}
	***F=29.416	*NSF*=1.550	*NSF*=2.332	**F=7.961
	***$F(B)$=59.400			
	***$F(I)$=16.707			
	***$F(BxI)$=7.141			

Results are expressed as the mean value ± SD (n=5). A two-way ANOVA was performed to determine the influence of both BC and inoculation treatment, on NUE in sunflower plants. The results are shown with the test statistic for each case (B—biochar level; I—inoculation treatment; BxI—biochar x inoculation treatment interaction). A one-way ANOVA was performed to determine the influence of inoculation treatment (C- non-inoculated; B- bacteria *P. reactans*; F- AMF *R. irregularis;* Mix - mixture of B/F) on NUE for each BC percentage. Means for inoculation treatments with different *letters* are significantly different from each other (P<0.05) given by the Duncan test. The test results are displayed as *NS*-Non-significant at the level P>0.05; *significant at the level P<0.05; **significant at the level P<0.01; ***significant at the level P<0.001.

3.1.4.2. Phosphorus (P)

Total P content in shoots (Figure 3.5 A) ranged from 88.15 to 155.55 g kg^{-1},while in roots (Figure 3.5 B) varied between 90.41 and 130 g kg^{-1}. Overall, increasing BC percentages lead to a generalized decrease in shoots' P accumulation. For instance, non-inoculated plants recorded an average reduction of 13, 24 and 38% in P accumulation in shoots at 2.5, 5 and 10% BC, respectively, when compared to the plants grown in non-amended soils. On the other hand, BC did not significantly (P>0.05) influence P accumulation in roots. In addition, microbial treatments seem to have a weak influence on the accumulation of P in both tissues.

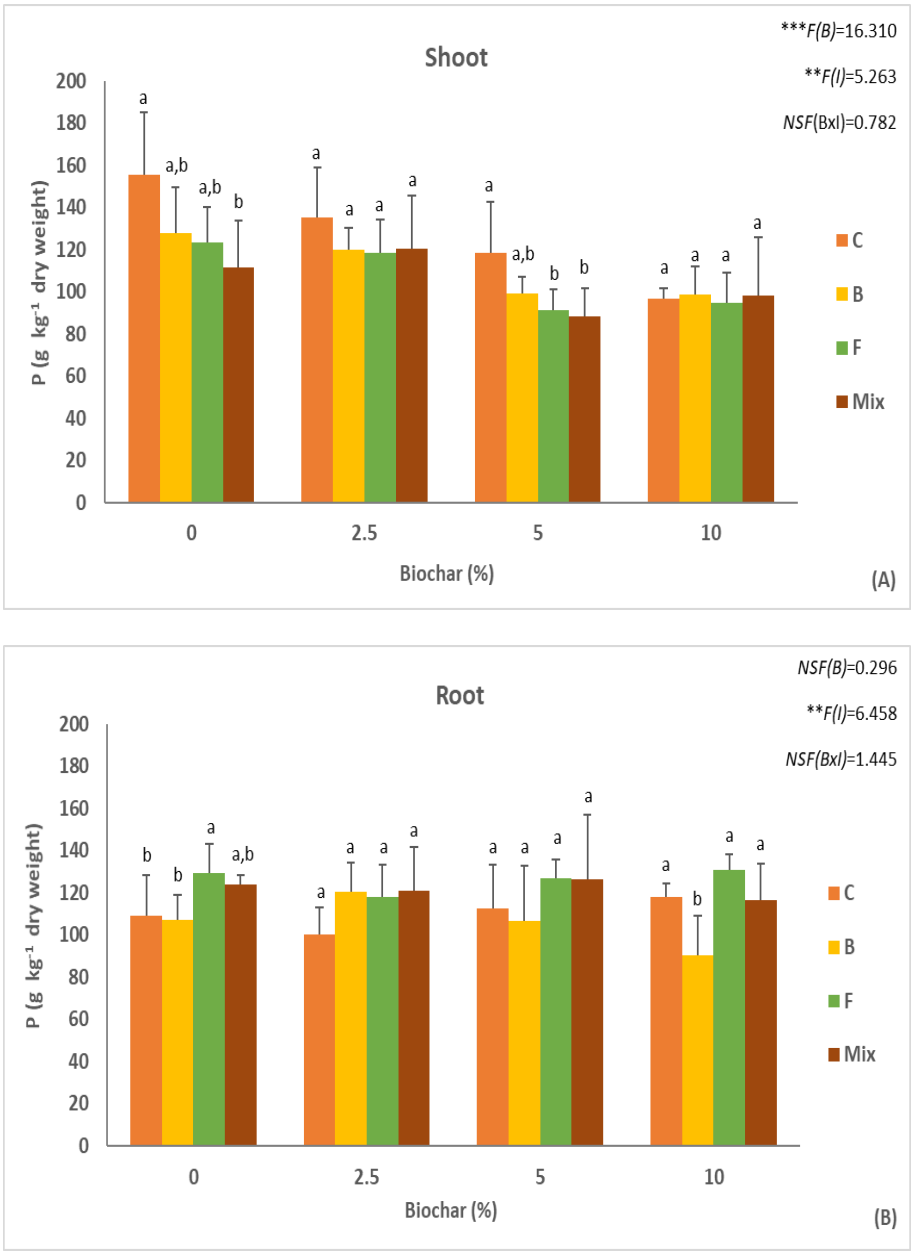


Figure 3.5 - Shoot (A) and root (B) phosphorus content of sunflower plants grown in a mining soil amended with different BC percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants (C - non-inoculated; B - bacteria *P. reactans*; F - AMF *R. irregularis*; Mix - mixture of B/F). Results are expressed as the mean value ± SD (n=5). A two-way ANOVA was performed to determine the influence of biochar and inoculation treatment, in shoot and root P content. The results are shown with the test statistic for each case (B- biochar level; I- inoculation treatment; BxI- biochar x inoculation treatment interaction). A one-way ANOVA was performed to determine the influence of inoculation treatment (C, B, F, Mix) on shoot and root P content for each BC percentage. Means for inoculation treatments with different *letters* are significantly different from each other *(P<0.05)* given by the Duncan test. For shoot P content, the values of one-way ANOVA are *F=3.356, *NSF*=0.749, *F=3.947 *and NSF*=0.059, and for root P content *F=3.316, *NSF*=1.871, *NSF*=1.038 and **F=7.688, respectively for 0, 2.5, 5 and 10% of BC. The results are

displayed as *NS*- Non-significant at the level *P*>0.05; *significant at the level *P*<0.05; **significant at the level *P*<0.01; ***significant at the level *P*<0.001.

Values for PUE (Table 3.3) varied between 7043.58 and 11171.07. In a general, BC addition enhanced PUE, with plants grown at 2.5, 5 and 10% BC, showing increases in average of 6, 24 and 41% respectively, when compared to the non-amended plants. Overall, bioinoculation, especially the Mix and F treatments, increased PUE both in presence and absence of BC. However, no significant differences were observed for the plants grown at 10% of BC.

Table 3.3 - Phosphorus Use Efficiency (PUE) in sunflower plants grown in a mining soil amended with different BC percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants at the end of the experiment.

Inocula	**Biochar %**			
	0	**2.5**	**5**	**10**
C	7043.58±1026.40[b]	7439.22±755.02[b]	8717.77± 1224.52[b]	9900.49±433.69[a]
B	8561.05±361.97[a]	8317.52±486.72[a,b]	10046.68±1054.05[a,b]	10380.60±1315.80[a]
F	8336.01±478.83[a]	8492.88±772.31[a]	10212.85±1004.28[a,b]	9975.35± 1287.30[a]
Mix	8487.90±853.59[a]	8965.77±717.16[a]	11171.07± 1068.66[a]	9502.29± 1351.25[a]
	***F*=4.775**	***F*=4.247**	***F*=4.281**	***NSF*=0.479**
		****F(B)*=24.019**		
		****F(I)*=7.062**		
		***NSF(BxI)*=1.522**		

Results are expressed as the mean value ± SD (n=5). A two-way ANOVA was performed to determine the influence of both BC and inoculation treatment, on PUE in sunflower plants. The results are shown with the test statistic for each case (B—biochar level; I—inoculation treatment; BxI—biochar x inoculation treatment interaction). A one-way ANOVA was performed to determine the influence of inoculation treatment (C- non-inoculated; B- bacteria *P.reactans*; F- AMF *R. irregularis;* Mix - mixture of B/F) on NUE for P, for each BC percentage. Means for inoculation treatments with different *letters* are significantly different from each other (*P*<0.05) given by the Duncan test. The test results are displayed as *NS*-Non-significant at the level *P*>0.05; *significant at the level *P*<0.05; **significant at the level *P*<0.01; ***significant at the level *P*<0.001.

3.1.5. Metal determination

3.1.5.1. Cu accumulation

The results for Cu content in sunflowers' shoots and roots are respectively presented in Figures 3.6 A and 3.6 B.

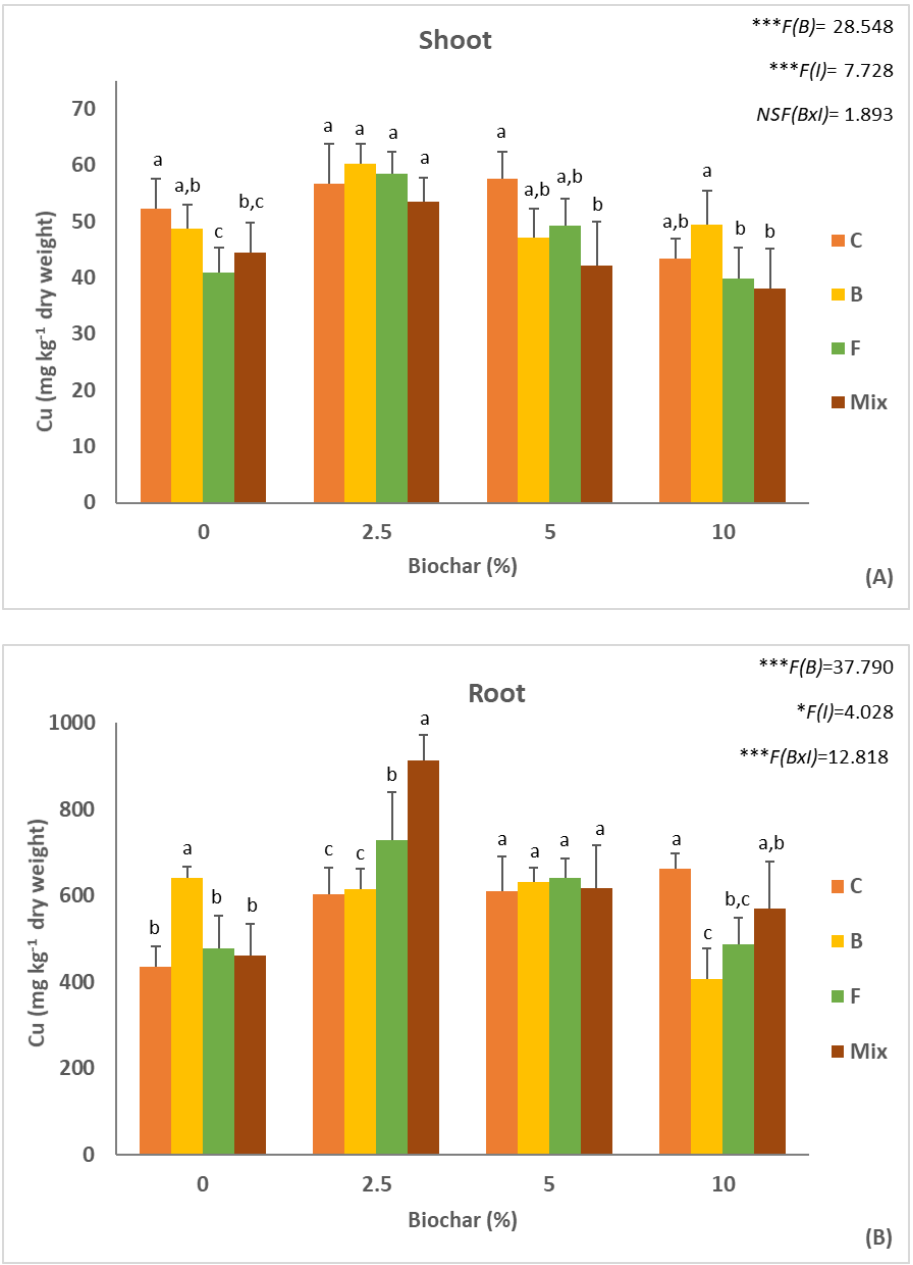


Figure 3.6 - Shoot (A) and root (B) copper content of sunflower plants grown in a mining soil amended with different BC percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants (C - non-inoculated; B - rhizobacteria *P. reactans*; F - AMF *R. irregularis*; Mix - mixture of B/F). Results are expressed as the mean value ± SD (n=5). A two-way ANOVA was performed to determine the influence of both biochar and inoculation treatment, in Cu accumulation on plant

shoots and roots. The results are shown with the test statistic for each case (B—biochar level; I—inoculation treatment; BxI—biochar x inoculation treatment interaction). A one-way ANOVA was performed to determine the influence of inoculation treatment (C, B, F, Mix) on Cu accumulation shoots and roots, for each BC percentage. Means for inoculation treatments with different *letters* are significantly different from each other (P<0.05) given by the Duncan test. For shoot Cu content, the values of one-way ANOVA are**F*=5.224, *NSF*=1.736, *NSF*=2.484 and **F*=3.937, and for root Cu content ****F*=12.418, ****F*=18.376, *NSF*=0.184 and ****F*=10.910, respectively for 0, 2.5, 5 and 10% of BC. The test results are displayed as *NS*-Non-significant at the level *P*>0.05; *significant at the level *P*<0.05; **significant at the level *P*<0.01; ***significant at the level *P*<0.001.

Copper accumulation in sunflower shoots (Figure 3.6 A) varied between 38.05 and 60.36 mg kg^{-1}, while in roots ranged from 406.48 to 912.97 mg kg^{-1} (Figure 3.6 B).Cu accumulation in both tissues was significantly (P<0.05) influenced by BC addition and by inoculation. Plants grown in soils amended with 2.5 and 5% of BC showed higher Cu accumulation in both tissues if compared to control plants. Indeed, it was observed an increment of 8 and 9% in shoots and of 28 and 29% in roots. In non-amended soil, Cu shoot accumulation was reduced by the inoculation of F and Mix, while in roots B treatment induced Cu accumulation. In BC amended soils, bioinoculants had a low impact on Cu accumulation in shoots, however in roots at 2.5% of BC the accumulation was highly increased (34%) by the inoculation of the rhizobacteria (B).

Table 3.4 - Shoot and root Cu bioconcentration (BCF) and translocation factor (TF) in sunflower plants grown in a mining soil amended with different BC percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants at the end of the experiment.

Biochar (%)	Inocula	BCF Cu		TF Cu
		Shoot	Root	
0	C	0.049±0.005^{a}	0.40±0.04^{b}	0.121±0.018^{a}
	B	0.045±0.004^{a,b}	0.59±0.02^{a}	0.076±0.005^{c}
	F	0.038±0.004^{c}	0.44±0.07^{b}	0.086±0.007^{b,c}
	Mix	0.041±0.005^{b,c}	0.43±0.07^{b}	0.090±0.009^{b}
		***F*=5.267**	****F*=12.417**	****F*=16.955**
2.5	C	0.053±0.007^{a}	0.56±0.06^{c}	0.094±0.008^{a,b}
	B	0.056±0.003^{a}	0.57±0.04^{c}	0.098±0.010^{a}
	F	0.054±0.004^{a}	0.67±0.10^{b}	0.082±0.014^{b}
	Mix	0.050±0.004^{a}	0.85±0.06^{a}	0.059±0.007^{c}
		***NSF*=1.733**	****F*=18.367**	****F*=19.911**
5	C	0.053±0.005^{a}	0.57±0.08^{a}	0.095 ±0.006^{a}
	B	0.044±0.005^{b}	0.58±0.03^{a}	0.075±0.005^{b}
	F	0.046±0.005^{b}	0.59±0.04^{a}	0.077±0.004^{b}
	Mix	0.039±0.007^{b}	0.57±0.09^{a}	0.069±0.012^{b}
		***F*=6.054**	***NSF*=0.184**	***F*=10.242**
10	C	0.040±0.003^{a,b}	0.61±0.03^{a}	0.066±0.006^{c}
	B	0.046±0.006^{a}	0.38±0.07^{c}	0.134±0.019^{a}
	F	0.037±0.005^{b}	0.45±0.06^{b,c}	0.082±0.011^{b}
	Mix	0.035±0.007^{b}	0.53±0.10^{a,b}	0.067±0.008^{c}
		***F*=3.934**	****F*=10.909**	****F*=37.139**
		****F(B)*=26.608*	****F(B)*=37.783*	****F(B)*=7.722*
		****F(I)*=9.723*	**F(I)*=4.025*	****F(I)*=27.920*
		**F(BxI)*=2.597*	****F(BxI)*=12.814*	****F(BxI)*=20.643*

Results are expressed as the mean value ± SD (n=5). A two-way ANOVA was performed to determine the influence of both BC and inoculation treatment, in the sunflower Cu bioconcentration and translocation factor. The results are shown with the test statistic for each case (B—biochar level; I—inoculation treatment; BxI—biochar x inoculation treatment interaction). A one-way ANOVA was performed to determine the influence of inoculation treatment (C- non-inoculated; B - bacteria P. reactans; F - AMF R. irregularis; Mix - mixture of B/F)

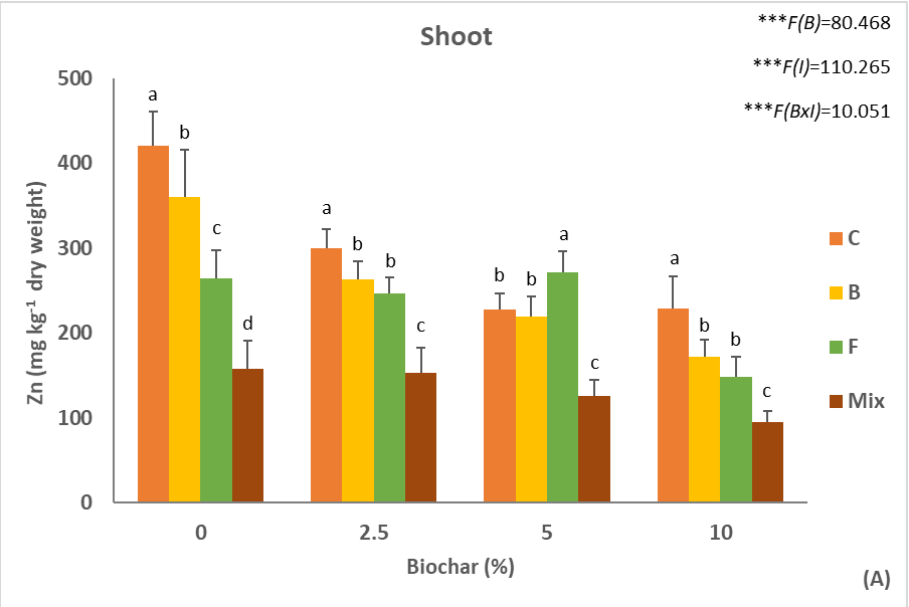
on Cu root and shoot BCF and TF, for each BC percentage. Means for inoculation treatments with different letters are significantly different from each other (P<0.05) given by the Duncan test. The test results are displayed as NS-Non-significant at the level P>0.05; *significant at the level P<0.05; **significant at the level P<0.01; ***significant at the level P<0.001.

The Cu BCF was higher for sunflowers' roots than for shoots (Table 3.4). According to the results of the two-way ANOVA, BC significantly (P<0.05) influenced Cu BCFs. In roots, increasing BC addition resulted in higher Cu BCF values, while no remarkable differences were observed for shoots. In general, microbial inoculation influenced Cu BCF in roots, since this factor was increased by 34% with the mixed inoculation at 2.5% of BC if compared to the non-inoculated plants. A different trend was observed at 10% of BC, where bioinoculants decreased roots' BCF.

Overall, BC increasing concentrations and bioinoculation significantly (P<0.05) decreased Cu TF.

3.1.5.2. Zn accumulation

The results for Zn content found in sunflowers' shoots and roots are respectively presented in Figures 3.7 A and 3.7 B.



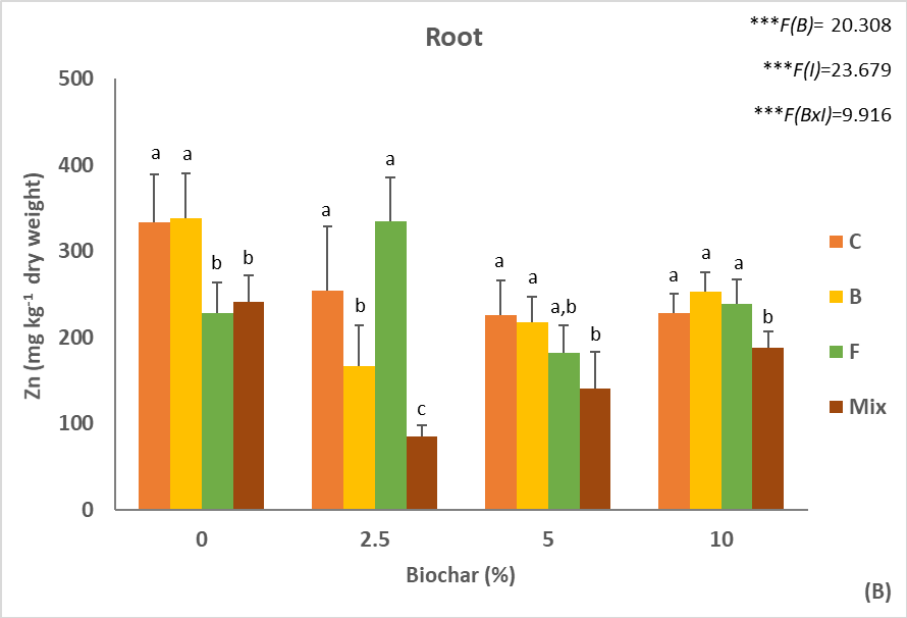


Figure 3.7 - Shoot (A) and root (B) Zn content of sunflower plants grown in a mining soil amended with different BC percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants (C - non-inoculated; B - rhizobacteria *P. reactans*; F - AMF *R. irregularis*; Mix - mixture of B/F) and biochar (0, 2.5, 5 and 10%). Results are expressed as the mean value ± SD (n=5). A two-way ANOVA was performed to determine the influence of both biochar and inoculation treatment, in Zn accumulation on plant shoots and roots. The results are shown with the test statistic for each case (B-biochar level; I-inoculation treatment; BxI-biochar x inoculation treatment interaction). A one-way ANOVA was performed to determine the influence of inoculation treatment (C, B, F, Mix) on Zn accumulation in shoots and roots, for each BC percentage. Means for inoculation treatments with different letters are significantly different from each other (P<0.05) given by the Duncan test. For shoot Zn content, the values of one-way ANOVA are ***F=38.163, ***F=33.956, ***F=41.148 and ***F=23.734, and for root Zn content **F=8.736, ***F=22.097, **F=5.588 and ***F=7.239, respectively for 0, 2.5, 5 and 10% of BC. The test results are displayed as NS-Non-significant at the level P>0.05; *significant at the level P<0.05; **significant at the level P<0.01; ***significant at the level P<0.001.

Zinc accumulation in sunflowers' shoots (Figure 3.7 A) varied between 94.58 and 421.08 mg kg^{-1}, while in roots ranged from 85.23 to 338.92 mg kg^{-1} (Figure 3.7 B). Zn accumulation in both plant tissues was significantly (P<0.05) influenced by BC addition. In a general way, increasing BC doses induced a decrease in Zn content in shoots. For instance, plants grown at 2.5% of BC recorded reductions in average of 29%, when compared to plants grown without BC addition. On the other hand, in roots no clear trend was observed for the BC addition. For instance, at 2.5 % of BC plants roots inoculated

with B treatment suffered a reduction of 35%, while the F inoculation induced an increase of 24% on Zn content found on roots, when compared to the non-inoculated plants.

Table 3.5 - Shoot and root Zn bioconcentration (BCF) and translocation factor (TF) in sunflower plants grown in a mining soil amended with different biochar percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants at the end of the experiment.

Biochar (%)	Inocula	BCF Zn		TF Zn
		Shoot	**Root**	
0	C	1.84±0.18[a]	1.46 ± 0.24[a]	1.41±0.22[a]
	B	1.58±0.24[b]	1.48± 0.23[a]	1.01±0.21[b]
	F	1.16±0.15[c]	1.00 ± 0.15[b]	1.06±0.13[b]
	Mix	0.69±0.14[d]	1.06 ± 0.14[b]	0.57±0.04[c]
		******F*=38.164**	*****F*=8.735**	******F*=33.035**
2.5	C	1.31±0.10[a]	1.12 ± 0.33[b]	1.08±0.18[b]
	B	1.15±0.10[b]	0.73± 0.21[c]	1.44±0.19[a]
	F	1.08±0.09[b]	1.47± 0.23[a]	0.75±0.16[c]
	Mix	0.67±0.13[c]	0.37± 0.06[d]	1.61±0.23[a]
		******F*=33.954**	******F*=22.097**	******F*=19.934**
5	C	1.00±0.08[b]	0.99± 0.18[a]	1.13±0.08[b]
	B	0.96±0.10[b]	0.95± 0.13[a]	1.03±0.20[b,c]
	F	1.19±0.11[a]	0.80± 0.14[a,b]	1.67±0.18[a]
	Mix	0.55±0.08[c]	0.62± 0.19[b]	0.85±0.13[c]
		******F*=41.157**	*****F*=5.588**	******F*=20.653**
10	C	1.00±0.17[a]	1.00± 0.10[a]	1.00±0.12[a]
	B	0.75±0.11[b]	1.11± 0.10[a]	0.68±0.06[b]
	F	0.65±0.11[b]	1.05± 0.13[a]	0.56±0.07[c]
	Mix	0.41±0.06[c]	0.82± 0.09[b]	0.51±0.09[c]
		******F*=23.734**	*****F*=7.239**	******F*=26.509**
		****F(B)*=80.468	****F(B)*=20.307	****F(B)*=61.818
		****F(I)*=110.269	****F(I)*=23.679	****F(I)*=21.289
		****F(BxI)*=10.052	****F(BxI)*=9.916	****F(BxI)*=25.657

Results are expressed as the mean value ± SD (n=5). One-way ANOVA was performed to determine the influence of inoculation treatment (C- non-inoculated; B- bacteria *P. reactans*; F-

AMF *R. irregularis;* Mix - mixture of B/F) on Zn BCFs and TF. Means for inoculation treatments with different *letters* are significantly different from each other (P<0.05) given by the Duncan test. The test results are displayed as *NS*-Non-significant at the level P<0.05; *significant at the level P<0.05; **significant at the level P<0.01; ***significant at the level P<0.001. Two-way ANOVA was performed to determine the influence of both biochar and inoculation treatment, in Zn bioconcentration and translocation factor. The results are shown with the test statistic for each case (B—biochar level; I—inoculation treatment; BxI—biochar x inoculation treatment interaction).

Zinc BCF in shoots varied between 0.41 and 1.84, while in roots ranged from 0.37 to 1.48 (Table 3.5). According to the results of the two-way ANOVA, Zn BCFs for shoots and roots were significantly (P<0.05) influenced by BC doses and by bioinoculation. Plants' shoots generally showed lower Zn BCF with the addition of increasing doses of BC, with non-inoculated plants at 2.5 and 5% of BC showing reductions of 29 and 46%, respectively, when compared to the non-inoculated plants grown in the absence of BC. The same scenario was observed in roots, with reductions of 23 and 32% recorded for non-inoculated plants at 2.5 and 5% of BC, respectively. On the other hand, the addition of most bioinoculants reduced Zn BCF in shoots, particularly at 10% of BC. A similar trend was observed in the roots of plants inoculated with the Mix inocula.

Zinc TF varied between 0.51 and 1.67 (Table 3.5). In general, BC addition induced reductions in this factor of 23, 20 and 29% respectively for non-inoculated plants treated with 2.5, 5 and 10% of BC. On the other hand, microbial treatments significantly (P<0.05) influenced Zn TF, in particular at 2.5% of BC, where plants inoculated with B and Mix, showed higher TF.

3.2. Soil analysis

3.2.1. Extractable Cu and Zn soil fractions

The water-extractable and NH_4-Ac-extractable soil fractions for Cu and Zn are displayed in Table 3.6.

Table 3.6 - Water - and NH_4-Ac-extractable Cu and Zn levels (mg kg^{-1}) in a mining soil amended with different BC percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants at the end of the experiment.

Biochar (%)	Inocula	Extractable soil Cu (mg kg^{-1})		Extractable soil Zn (mg kg^{-1})	
		H_2O	**NH_4-Ac**	**H_2O**	**NH_4-Ac**
0	C	0.68 ± 0.04^{c}	12.01±0.19^{a,b}	0.31±0.10^{c}	2.43± 0.33^{b}
	B	1.04 ± 0.08^{b}	13.05±0.29^{a}	0.56±0.12^{b}	2.00± 0.15^{a}
	F	1.47 ± 0.09^{a}	10.88±0.80^{b}	1.02±0.19^{a}	2.04± 0.03^{a}
	Mix	1.44 ± 0.08^{a}	11.18±1.79^{b}	0.38±0.06^{c}	2.07± 0.14^{a}
		******F*=122.113**	****F*=4.767**	******F*=22.020**	*****F*=5.968**
2.5	C	0.88 ± 0.08^{c}	18.95±1.15^{b}	0.92±0.13^{a}	1.63± 0.16^{a}
	B	1.24 ± 0.14^{b}	20.03±1.08^{b}	0.86±0.13^{a}	1.93± 0.17^{b}
	F	1.31 ± 0.07^{b}	19.73±0.91^{b}	0.44±0.13^{c}	1.96± 0.23^{b}
	Mix	1.65 ± 0.06^{a}	21.53±1.08^{a}	0.62±0.12^{b}	1.94± 0.31^{b}
		******F*=57.514**	****F*=5.213**	******F*=15.527**	***NSF*=2.614**
5	C	0.85 ± 0.14^{d}	21.10±0.68^{a}	0.79±0.21^{a}	1.64± 0.11^{a}
	B	1.12 ± 0.11^{c}	21.40±1.32^{a}	0.39±0.07^{b}	1.87± 0.15^{b}
	F	1.38± 0.12^{b}	18.80 ±0.89^{b}	0.78±0.12^{a}	1.96± 0.13^{b}
	Mix	1.55 ± 0.05^{a}	16.41 ±1.34^{c}	0.38±0.18^{b}	1.96± 0.27^{b}
		******F*=39.424**	******F*=22.426**	*****F*=8.908**	****F*=4.301**
10	C	0.98 ± 0.09^{d}	16.20±1.90^{a}	0.37±0.11^{a}	2.13± 0.32^{b}
	B	1.23 ± 0.13^{c}	16.40±1.32^{a}	0.25±0.08^{a}	1.98± 0.20^{b}
	F	1.41 ± 0.12^{b}	15.95±1.47^{a}	0.24±0.04^{a}	1.60± 0.13^{a}
	Mix	1.59 ± 0.05^{a}	13.74±1.02^{b}	0.05±0.02^{b}	1.65± 0.22^{a}
		******F*=33.152**	****F*=3.567**	******F*=39.229**	*****F*=6.688**
		****F(B)*=8.701	****F(B)*=215.992	****F(B)*=109.468	****F(B)*=9.488
		****F(I)*=208.130	****F(I)*=11.120	****F(I)*=27.045	*NSF(I)*=0.381
		****F(BxI)*=4.040	****F(BxI)*=7.334	****F(BxI)*=22.982	****F(BxI)*=6.184

Results are expressed as the mean value ± SD (n=5). A two-way ANOVA was performed to determine the influence of both BC and inoculation treatment, in the Cu and Zn extractable soil fractions. The results are shown with the test statistic for each case (B—biochar level; I—inoculation treatment; BxI—biochar x inoculation treatment interaction). A one-way ANOVA was performed to determine the influence of inoculation treatment (C- non-inoculated; B - bacteria *P. reactans*; F- AMF *R. irregularis;* Mix - mixture of B/F) on Cu and Zn extractable soil fractions, for

each BC percentage. Means for inoculation treatments with different *letters* are significantly different from each other *(P*<0.05) given by the Duncan test. The test results are displayed as *NS*-Non-significant at the level *P*>0.05; *significant at the level *P*<0.05; **significant at the level *P*<0.01; ***significant at the level *P*<0.001.

The water-extractable Cu soil fractions ranged from 0.68 to 1.65 mg kg^{-1}, while NH_4-Ac-extractable fractions varied from 10.88 to 21.53 mg kg^{-1} (Table 3.6). Biochar addition at 2.5, 5 and 10% increased soil water-extractable forms by 23, 20 and 31%, respectively. A similar trend was observed for NH_4-Ac extractable fractions, particularly in non-inoculated soils amended with 2.5 and 5% of BC. Bioinoculation, in particular Mix and F treatments, influenced positively Cu water-extractable fractions, inducing an increase of 45 and 38%, respectively at 5% of BC. However, overall microbial inoculants tended to decrease NH_4-Ac-extractable Cu fractions.

The water-extractable Zn soil fractions ranged from 0.05 to 1.02 mg kg^{-1}, and NH_4-Ac-extractable fractions varied between 1.60 and 2.43 mg kg^{-1} (Table 3.6). Generally, the highest water-extractable Zn levels were recorded at 2.5 % of BC. On the other hand, at 5 and 10% of BC it was observed a decrease of 33 and 12% in NH_4-Ac-extractable fractions, respectively. Microbial inoculants did not significantly (P>0.05) influence NH_4-Ac-extractable fractions.

3.2.2. Soil microbial community analysis

DGGE analysis was performed for the rhizospheric samples taken for the three experimental time points (e.g. t_0, t_1 and t_f) as previously described. The first DGGE profile was composed by the non-inoculated samples and samples with bacterial inoculation (Figure 3.8), while the second DGGE profile was composed respectively by the inoculated samples with the AMF and with the mixed inocula (Figure 3.9).

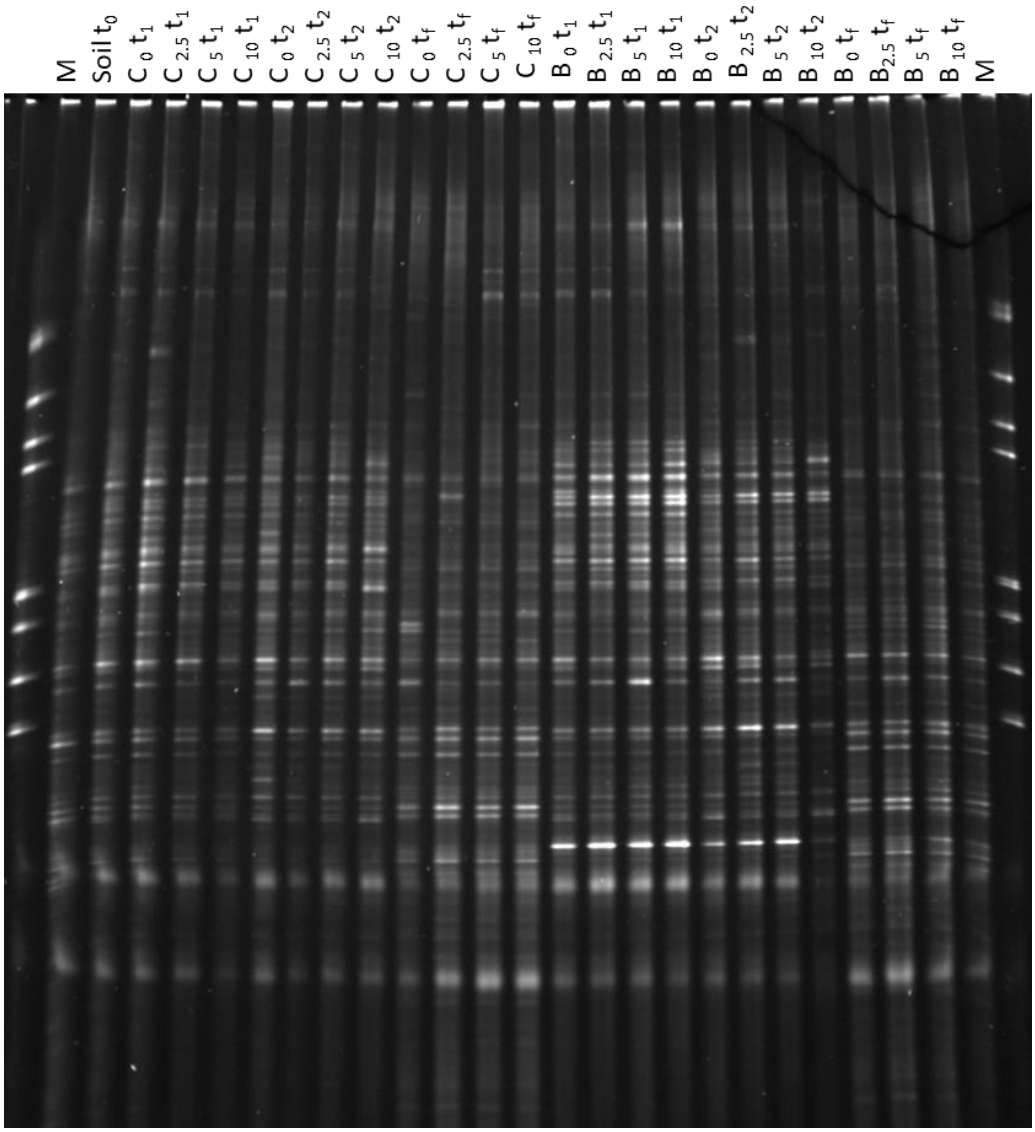


Figure 3.8 - DGGE profiles for the mining soil amended with different BC percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants (C- non-inoculated and B- *P. reactans*). Lanes M – Marker, as decribed in Henriques et al.(2004); Samples with t_1, t_2 and t_f were taken after the first and second inoculation and at the end of the experiment, respectively.

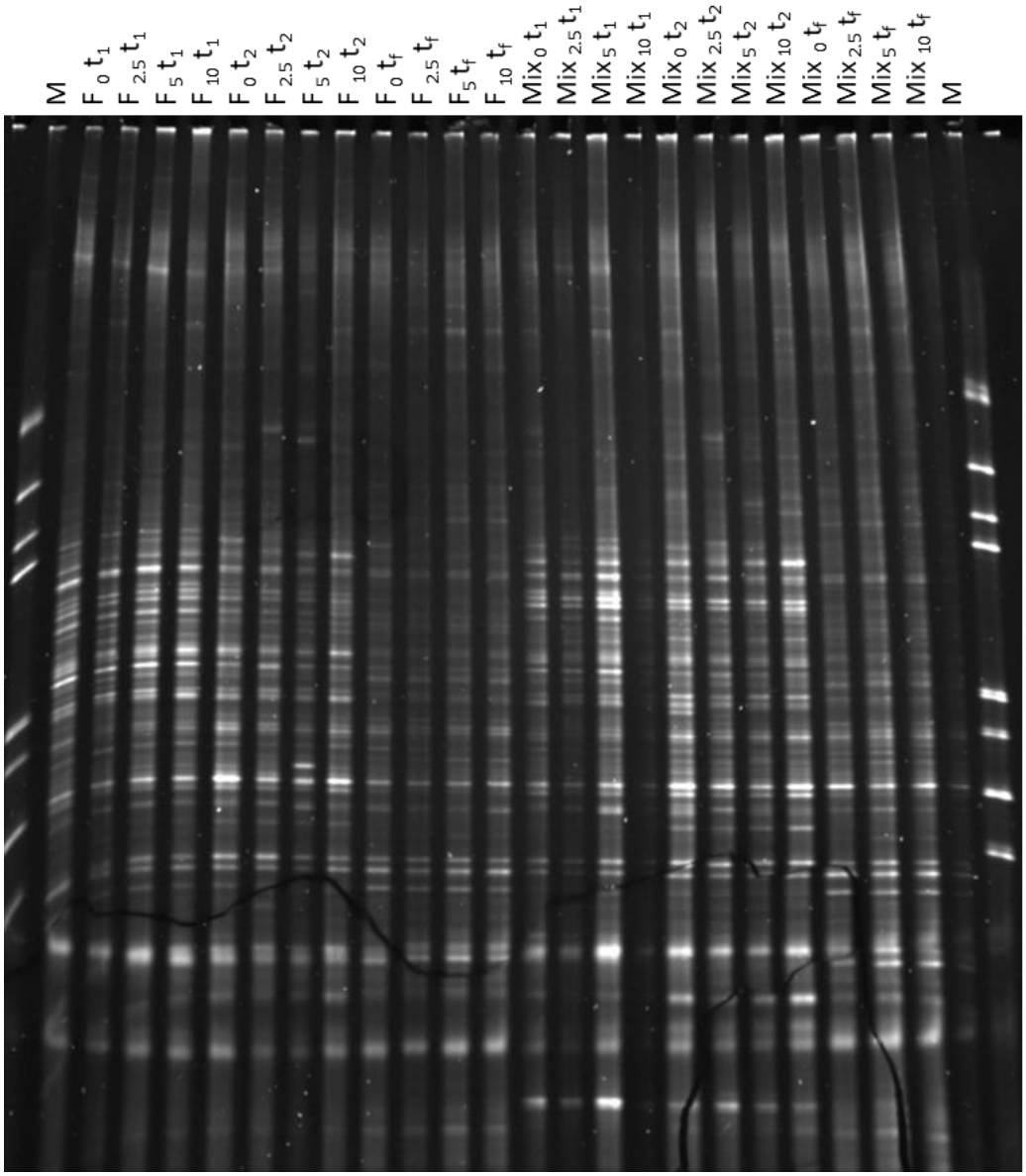


Figure 3.9 - DGGE profiles for the mining soil amended with different biochar percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants (F- AMF *R. irregularis;* Mix - mixture of B/F). Lanes M – Marker as decribed in Henriques et al. (2004) ; Samples with t_1, t_2 and t_f were respectively taken after the first and second inoculation and at the end of the experiment, respectively.

Clustering analysis showed differences among the bacterial community found on the rhizosphere soil samples (Figure 3.10).

In general, bacterial communities clustered into groups according to microbial inoculants, regardless the BC percentage applied on the soil. Additionally, clustering typically occurred in accordance with the different experiment time points; for instance, rhizosphere samples taken at t_1 and t_2 appeared to be frequently in the same group, therefore being more distant from the samples taken at t_f. Bacterial communities clustered into two major groups, which were closely related with the samples treated as control. Due to an error performed in loading stage, Mix $_{10}$ t_1 and Mix $_{10}$ t_f samples are more closely related between them than with the main group. Taking into account the observed distribution, a cluster analysis was conducted to compare the DGGE profiles obtained for communities in each sampling time (t_1, t_2 and t_f; Figure 3.11).

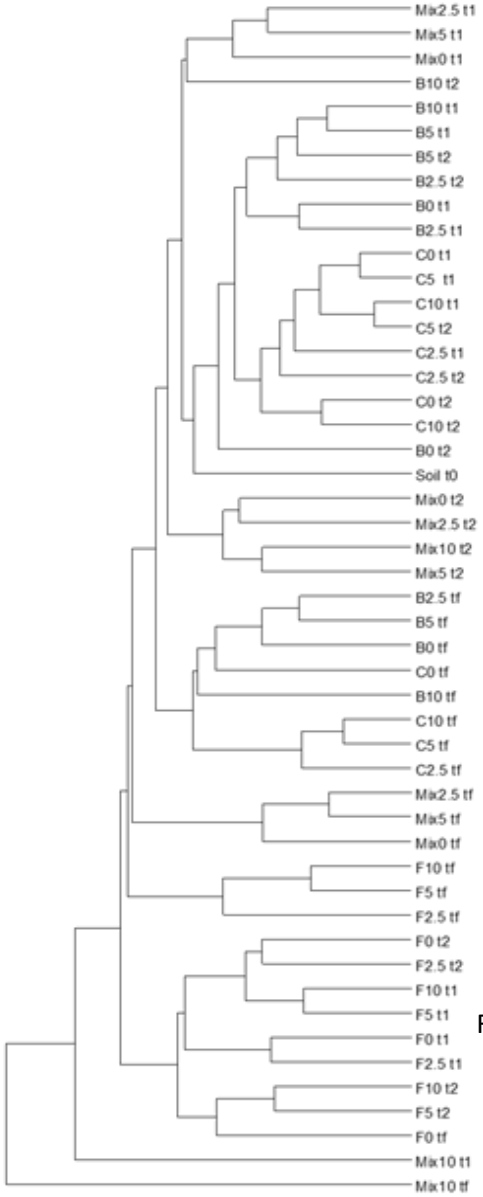
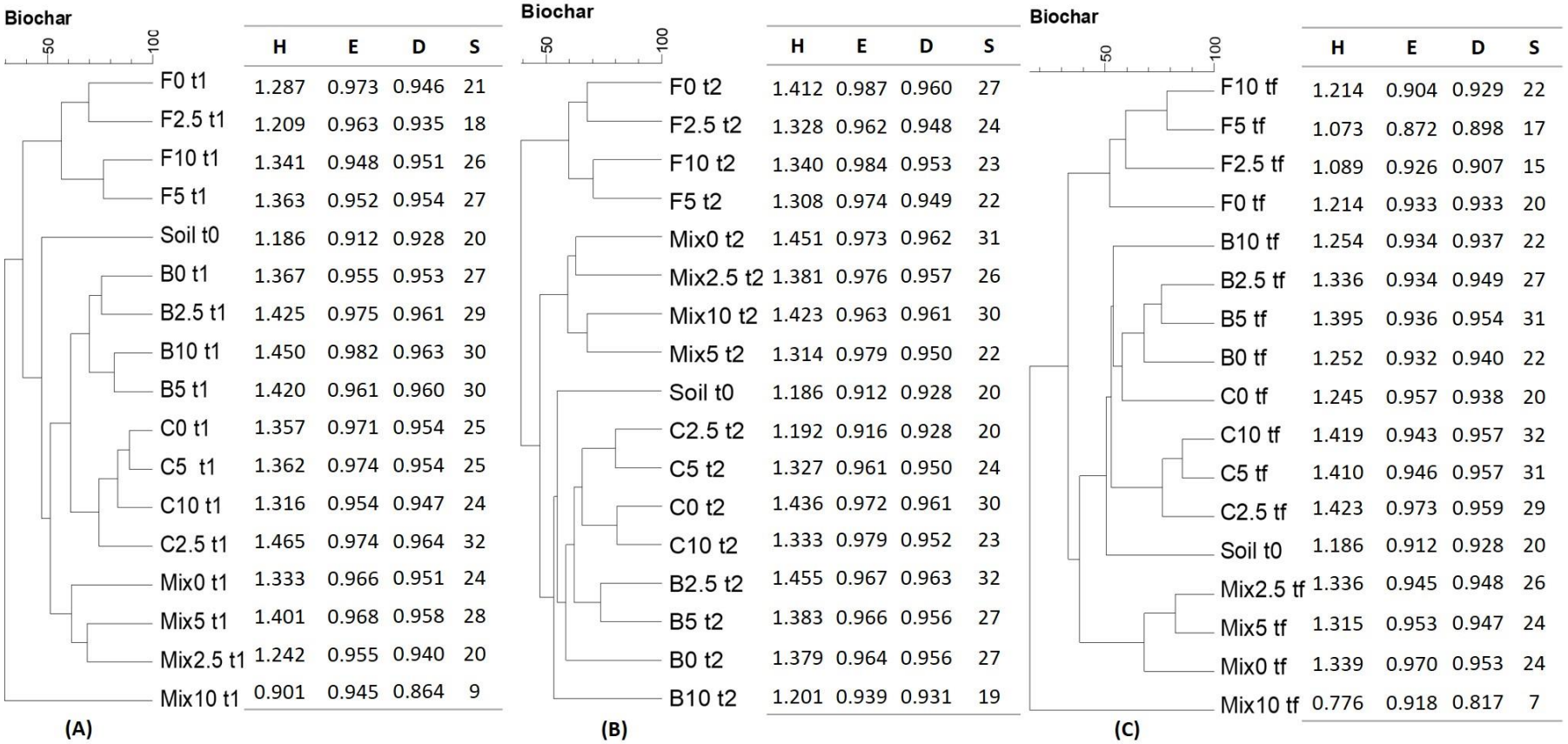


Figure 3.10 - Similarity of the rhizosphere soil samples based on the UPGMA clustering method. Soil was amended with different BC percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants (B- *P. reactans*; F- AMF *R. irregularis*; Mix - mixture of B/F). *Soil* t_0 and *C* - non inoculated soil; t_1 - after the first inoculation; t_2 - after the second inoculation; t_f - end of the experiment.



	H	E	D	S
F0 t1	1.287	0.973	0.946	21
F2.5 t1	1.209	0.963	0.935	18
F10 t1	1.341	0.948	0.951	26
F5 t1	1.363	0.952	0.954	27
Soil t0	1.186	0.912	0.928	20
B0 t1	1.367	0.955	0.953	27
B2.5 t1	1.425	0.975	0.961	29
B10 t1	1.450	0.982	0.963	30
B5 t1	1.420	0.961	0.960	30
C0 t1	1.357	0.971	0.954	25
C5 t1	1.362	0.974	0.954	25
C10 t1	1.316	0.954	0.947	24
C2.5 t1	1.465	0.974	0.964	32
Mix0 t1	1.333	0.966	0.951	24
Mix5 t1	1.401	0.968	0.958	28
Mix2.5 t1	1.242	0.955	0.940	20
Mix10 t1	0.901	0.945	0.864	9

	H	E	D	S
F0 t2	1.412	0.987	0.960	27
F2.5 t2	1.328	0.962	0.948	24
F10 t2	1.340	0.984	0.953	23
F5 t2	1.308	0.974	0.949	22
Mix0 t2	1.451	0.973	0.962	31
Mix2.5 t2	1.381	0.976	0.957	26
Mix10 t2	1.423	0.963	0.961	30
Mix5 t2	1.314	0.979	0.950	22
Soil t0	1.186	0.912	0.928	20
C2.5 t2	1.192	0.916	0.928	20
C5 t2	1.327	0.961	0.950	24
C0 t2	1.436	0.972	0.961	30
C10 t2	1.333	0.979	0.952	23
B2.5 t2	1.455	0.967	0.963	32
B5 t2	1.383	0.966	0.956	27
B0 t2	1.379	0.964	0.956	27
B10 t2	1.201	0.939	0.931	19

	H	E	D	S
F10 tf	1.214	0.904	0.929	22
F5 tf	1.073	0.872	0.898	17
F2.5 tf	1.089	0.926	0.907	15
F0 tf	1.214	0.933	0.933	20
B10 tf	1.254	0.934	0.937	22
B2.5 tf	1.336	0.934	0.949	27
B5 tf	1.395	0.936	0.954	31
B0 tf	1.252	0.932	0.940	22
C0 tf	1.245	0.957	0.938	20
C10 tf	1.419	0.943	0.957	32
C5 tf	1.410	0.946	0.957	31
C2.5 tf	1.423	0.973	0.959	29
Soil t0	1.186	0.912	0.928	20
Mix2.5 tf	1.336	0.945	0.948	26
Mix5 tf	1.315	0.953	0.947	24
Mix0 tf	1.339	0.970	0.953	24
Mix10 tf	0.776	0.918	0.817	7

Figure 3.11 – Similarity of the rhizosphere soil samples based on the UPGMA clustering method: partial dendrogram of the initial soil and the samples taken **(A) –** after the first inoculation *(t_1)*, **(B) –** after the second inoculation *(t_2)* and **(C) –** at the end of the experiment (t_f). Soil was amended with different biochar percentages (0, 2.5, 5, 10%) and treated with different microbial inoculants (B- *P. reactans*; F- AMF *R. irregularis*; Mix - mixture of B/F). *Soil t_0 and C* - non inoculated soil. For each sample three indexes were calculated represented by the letters H, E and D. The letters respectively represent H - Shannon's diversity index, E - equitability (evenness), D - Simpson's diversity index and S - number of bands recorded for each profile.

Important to notice that the initial soil (i.e. soil t_0) is more closely related to the communities with bacterial inoculation and the non-inoculated ones, than to the communities inoculated with AMF (Figure 3.11 (A) to (C)). A similar tendency occurred for the three time points, since the control samples are more related to the samples inoculated with the rhizobacteria (B). In addition, inoculation with AMF seemed to have a higher influence on the bacterial communities.

The Shannon index (H) revealed (Figure 3.11) that approximately more than half of samples tended to suffer a reduction in diversity throughout the experiment. Shannon's equitability (E) index followed a similar tendency, where approximatively 82% of the rhizospheric samples tended to show lower values for equitability at the end of the experiment, when in comparison to the beginning. Simpson diversity index (D) varied in accordance with the other indexes. In a general way, the number of bands found in each sample at the end of the experiment, represented by the letter S, tended to be lower than the number of bands found in each rhizospheric sample after the first inoculation (t_1). Indeed, this tendency was more expressive in the rhizospheric samples taken for the AMF inoculated plants.

The rhizospheric samples taken for the rhizobacteria (B) inoculated plants suffered an overall reduction of diversity, from the beginning until the end of the experiment. On the other hand, these samples appeared to have a higher diversity after the first inoculation, when compared to the non-inoculated samples. Conversely, lower bacterial diversity were found in rhizospheric samples with AMF inoculation if compared to non-inoculated samples. The samples that were co-inoculated with EDP28 and AMF had a tendency to maintain both Shannon and Simpson indexes throughout the experience.

To better understand the relationship between the samples, a matrix was retrieved from Bionumerics software (version 6.6, Applied Maths, St.-Martens-Laten, Belgium), based on the band intensity found for each DGGE profile. Using the Bray-Curtis dissimilarity index, an MDS plot was calculated and displayed in Figure 3.12.

Results confirmed a clear separation between samples inoculated with AMF from the remaining samples. It was also clear that the communities inoculated with the

rhizobacteria (B) grouped in close proximity with the communities that were not inoculated.

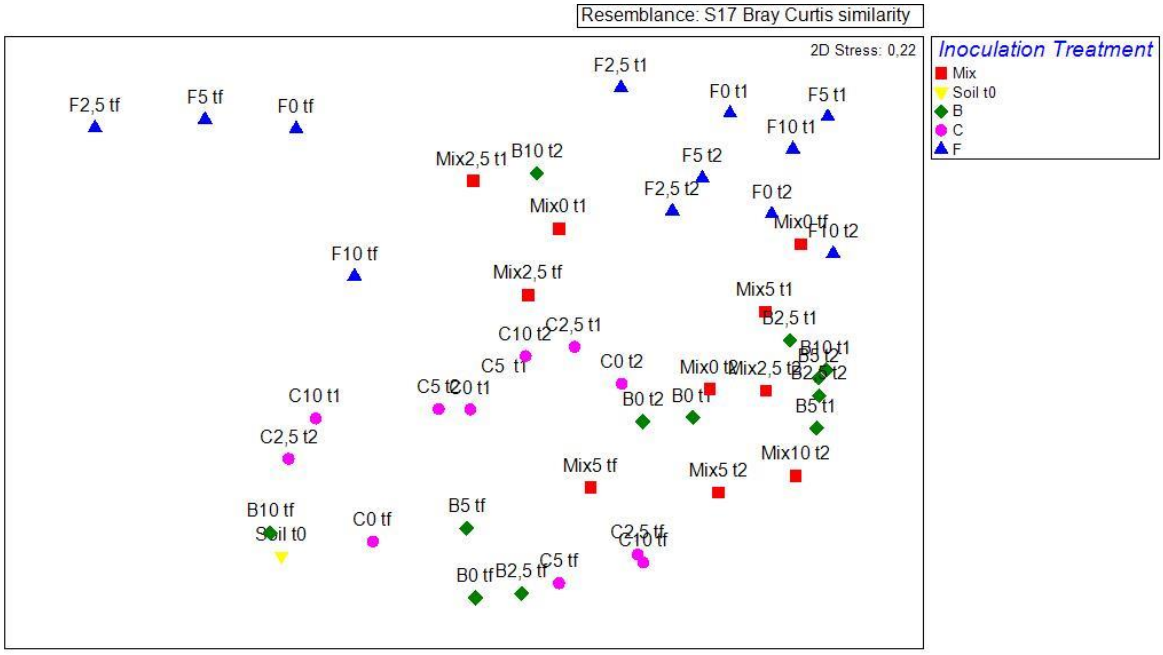


Figure 3.12 - MDS plot showing similarity between 47 samples collected along the experiment, as determined by DGGE fingerprinting analysis (stress value of 0.22). The outliers Mix $_{10}$ t_1 and Mix $_{10}$ t_f are not displayed in this MDS plot. B, *P. reactans*; F, AMF *R. irregularis*; Mix, mixture of B/F. Soil t_0 and C, non-inoculated soil.

4. Discussion

Soil pollution with metals can lead to the deterioration of soil properties, thus affecting soil health at a global scale due to metal persistence in the environment (Burges et al., 2018; Eugenio et al., 2018).

In this study, a mine metal-contaminated soil was amended with BC, a carbon-rich product obtained by biomass pyrolysis (Nartey and Zhao, 2014), at different percentages and inoculated with several beneficial microorganisms. The results of this study showed that the application of bioinoculants reduced the harmful effects of metals in *H. annuus* growth, by increasing biomass production and balancing nutrient uptake. Valuable to note this is one of the few studies that reported single and synergetic action of BC and bioinoculants, for assisted-phytoremediation purposes.

According to the Canadian Soil Quality Guidelines, the mining soil used in this work has concentrations of As, Cd, Cu and Pb (34.98 mg As kg^{-1}, 1.45 mg Cd kg^{-1}, 1080 mg Cu kg^{-1} and 71.06 mg Pb kg^{-1}, respectively) above the limit recommended for agricultural soils (12 mg As kg^{-1}, 1.4 mg Cd kg^{-1}, 63 mg Cu kg^{-1} and 70 mg Pb kg^{-1}) (CCME, 2018). However, Zn levels (e.g. 228.24 mg Zn kg^{-1}) were below the reference values for agricultural land use (250 mg Zn kg^{-1}) (CCME,2018). In addition, the target values for these metals according to the Dutch Standards were 29 mg As kg^{-1}, 0.8 mg Cd kg^{-1}, 36 mg Cu kg^{-1}, 85 mg Pb kg^{-1} and 140 mg Zn kg^{-1} (VROM, 2000).

In the present work, the application of increasing BC doses, in particular 2.5 and 5%, in a metal-contaminated soil generally tended to reduce considerably sunflowers' plants weight (i.e. shoot and root biomass), as well s shoot elongation. A different trend was observed by Ogundiran et al. (2018) in *Moringa oleifera* plants grown in Pb-contaminated soils amended with two types of BC (rice husk and groundnut shell BC), since rice-husk BC had a positive effect on *M. oleifera* plant's biomass, particularly on shoot yield. Another study showed that BC applied at 5% was capable to mitigate metal toxicity in the soil for *P. vulgaris* development, due to its ability to retain contaminants dispersion and consequently their accumulation in plant tissues (Lomaglio et al., 2018). In this study, the reduction of growth observed in plants cultivated in a soil amended with 2.5 and 5% of BC was probably due to the higher metal accumulation found in sunflower

tissues. Indeed, Mahar et al. (2016b) showed that Cu uptake by Chinese cabbage roots suffered a significative increase with the addition of a soil amendment (e.g. CaO) at a 5% rate. However, this treatment induced a significative reduction of plant dry biomass ratio that are in agreement with the results of the present study. Another study also showed reduced values for maize biomass after the addition of amendments due to higher As uptake by these plants (Rocco et al., 2018).

In the present study, sunflower plants amended with 10% of BC did not suffer a decrease in shoot and root biomass, unlike the plants treated with 2.5 and 5%. Moreover, Zn and Cu accumulation was lower in these plants when compared to plants grown without BC. Nonetheless, a previous study showed that the amendment with 10% of BC and co-inoculated with manure had a positive effect on spinach root biomass; the latter treatment also proved to enhance Cu accumulation in spinach shoots (Tahir et al., 2018).

Plants grown at 10% of BC also showed enhanced values for NUE. Higher NUE values may be connected to the maintenance of biomass values, since these plants were more efficient in N uptake and subsequent translocation to roots and shoots, resulting in enhanced crop yield/biomass production (Baligar et al., 2001). In addition, the plants amended with 2.5% of BC showed signs of chlorosis (Figure 4.1 A), which can be described as yellowish discoloration of parts of the leaf, as a result of N deficiency, once this nutrient is a constituent of chlorophyll (Roy et al., 2006). On the contrary, the plants amended with 10% of BC did not show these signs; instead, its greenness can be account as an indication of active growth (Roy et al., 2006) (Figure 4.1 B). A possible explanation for these results is the higher metal accumulation found on sunflower plants amended with BC at 2.5 and 5%, which can be associated to the negative symptoms observed in these plants leaves. Conversely, these signs were not observed in sunflower plants treated with 10% of BC, were metal accumulation was perhaps less pronounced.

Figure 4.1 - Sunflower plants taken at the end of the pot experiment showing chlorosis symptoms (A) for sunflower plants grown on soil amended with 2.5% of BC, while sunflower plants did not show this signs when amended with 10% of BC (B).

Chlorophyll content has a nearby connection to plant nutritional status (Konica Minolta, 2017), giving indications for several plant related aspects, such as leaf N content, plant photosynthetic capacity or even plant general health (Ling et al., 2011). The present work revealed that increasing BC levels usually induced lower chlorophyll content in sunflower leaves, particularly for plants grown at 2.5% of BC, when compared to the other amended and non-amended plants. These findings are in accordance with the results obtained for the biometric values. However, a different trend was observed by Ma et al. (2017) who showed that Zn application in wheat plants grown under water stress conditions was able to enhance SPAD values.

Bioinoculation attenuated the negative effects of BC, since inoculated plants showed higher biomass values than non-inoculated ones, for the same doses of BC, as well as higher chlorophyll contents. According to Ali et al. (2017) sorghum inoculation with *Streptomyces pactum* (Act12) enhanced shoot and root dry weights, as well as the chlorophyll content. Indeed, sunflower plants inoculated with the rhizobacteria *P. reactans* (B) usually showed higher shoot elongation, especially plants amended with 2.5 and 5% of BC. This strain exhibited benefic traits connected to plant growth promotion, such as IAA production and ACC-deaminase activity (Moreira et al., 2016b). Several

hormones including auxins, gibberellins and cytokinins can help plants to cope with adverse conditions, for instance with metal stress (Gangwar et al., 2014). According to Glick (2014) the majority of plants linked to ACC-deaminase PGPB producers' have longer shoots and roots, and have a greater ability to grow under unfavorable conditions (e.g. ethylene-inducing stress). For example, several bacterial strains with high ACC-deaminase activity were capable to enhance white clover shoot elongation (Pereira et al., 2015). On the other hand, several *Pseudomonas* strains have been studied for their capacity to colonize the rhizosphere (e.g. *P. putida* and *P. chlororaphis*), by providing substances that are directly connected to plant growth promotion (Venturi, 2006). For instance, Nadeem et al. (2014) reported that some *Pseudomonas* species are able to lower ethylene concentration by ACC-deaminase enzyme and decrease the availability of Na^+ with exopolysaccharides production.

In the present work, mix inoculation with PGPR and AMF resulted in a higher increase of shoots' elongation and biomass, when compared to the results obtained with single inoculation (e.g. B and AMF inocula) for the same parameters. Nadeem et al. (2014) showed that PGPR and AMF capable to tolerate stress conditions might improve plant survival and growth. Similarly Pérez-De-Luque et al. (2017) also studied the combined effect of the AMF *R. irregularis* and the rhizobacteria *P. putida* KT2440 on two different wheat cultivars with different abilities to form mycorrhiza. Both inocula had a positive impact on wheat growth. In the present study, the mixed inoculation with *R. irregularis* and the rhizobacteria *P. reactans* obtained the greatest results for shoot fresh biomass regardless BC percentage. These results are in agreement with Moreira et al. (2016a) that verified co-inoculation of maize plants with these beneficial microorganisms promoted a higher increase of shoots' biomass. This tendency is also in accordance with the results obtained by Mani et al. (2015), where co-inoculation with an AMF and a *Pseudomonas putida* strain proved to improve phytoremediation potential of sunflower in a Cd/Zn contaminated soil, as well as their synergetic beneficial effect in plant dry biomass.

Plant-microbe interactions showed the potential for microorganisms to facilitate/assist phytoremediation through their ability to accumulate metals, thus stimulating metal uptake and enhanced plant growth (Glick, 2010). The present work

demonstrated that bioinoculation, especially mixed inocula, was able to generally reduce metal content in sunflower tissues. Conversely, according to Hassan et al. (2013) inoculation with AMF *R. irregularis* promoted Cd and Zn accumulation in sunflower shoots, but also enhanced phytoextraction of Cd. Indeed, the plants inoculated with *R. irregularis* recorded Cd BCF > 1, showing the potential of AMF to promote Cd transportation from the contaminated soil to sunflower shoots.

Nitrogen can be entitled as the most essential nutrient necessary for plant development, as N deficiency can cause the delay of leaf growth and shoot elongation (Presterl et al., 2002). The present study showed that N was more mobilized for above ground tissues, since N accumulation was usually higher in sunflower shoots than in roots. Previous works demonstrated that BC was involved in nutrients adsorption (Ding et al., 2016), once it can act as a nutrient provider, by increasing the amount of nutrients available for plant uptake (Graber and Elad, 2013). Soil amended with BC can also induce alterations in soil capacity to retain water (Liu et al., 2017). Alburquerque et al. (2014) reported that BC with a higher ash content (e.g. wheat straw) was able to deliver a larger amount of nutrients in a plant-available form, thus influencing sunflower growth in a greater extent. The mine soil used in this experiment was acid, however its pH increased after BC addition, since the BC pH ranged from 8 to 10. For this reason, a plausible mechanism proposed for increased nutrient availability mediated by BC, might be the cationic exchange (Taghizadeh-Toosi et al., 2012). However, increased BC levels induced for instance an overall reduction of N allocation in sunflower shoots. A possible explanation for this situation is that this nutrient perhaps could be mobilized for other plant functions, such as synthesis of key cellular components like proteins (Morgan and Connolly, 2013), therefore sunflower shoots recorded reduced N levels. Biochar addition proved to also have a positive influence on NUE, except for plants amended with 2.5% of BC. As seen before, plants treated with this percentage of BC showed reduced biometric parameters, in particular shoot and root biomass, and plant height. This is probably related to the amount of available nutrients in the soil, which may not be sufficient for sunflower N demands, or conversely this plant was not capable to obtain the required nutrient from the soil, since metal accumulation induce a stress response for the plants

amended with 2.5% of BC. Jilling et al. (2018) suggests that the interaction between root exudates and microbes can increase the mineral-associated N fraction bioavailable for plant uptake. On the other hand, bioinoculation with AMF appeared to reduce plant N uptake, particularly in sunflower shoots. These results are in agreement with Moreira et al. (2016a) who referred that N uptake by plants could be affected by the presence of AMF, once it requires high levels of this nutrient for its maintenance. The co-inoculation with PGPR and AMF resulted in enhanced N uptake and NUE by sunflower roots, which are in accordance with the results obtained in previous works (Garg and Chandel, 2010).

Besides being an essential nutrient for several plant processes, such as photosynthesis and root growth, P is also crucial for optimal crop yield (Dugdug et al., 2018). In this study, plants amended with increasing levels of BC were negatively correlated to P content in sunflower shoots. These results are not in agreement with Salim (2016), that demonstrated that BC amended at 5% enhanced P content in wheat plants. After BC addition, the mine soil pH is expected to increase along with the proportion of HPO_4^{2-} that is available for P uptake by plants (Roy et al., 2006). A previous study showed that the effects of increasing BC doses on P sorption was positively correlated to the level of soil acidity (Xu et al., 2014). Some microorganisms can also be used as P suppliers for plants as a cost-effective alternative to phosphatic fertilizers (Suleman et al., 2018), since they are capable to solubilize P being entitled as phosphate-solubilizing microorganisms (PSB) (Chandra and Singh, 2016). A previous work revealed that *Pseudomonas* sp. EAV can be used as PSB in order to enhance maize growth on P-deficient soils (Pereira and Castro, 2014). Indeed, according to Merlos et al. (2016) sunflower plants treated with the AMF inocula showed particularly higher P content in sunflowers' roots specially in the absence of BC. Our results showed higher levels of N and P in inoculated plants with AMF than in non- inoculated ones regardless the level of metal soil contamination. In general, increasing BC levels, as well as, the presence of AMF and mixed inoculation also promoted PUE. According to Rose et al. (2013), plants with higher PUE normally have lower shoot P contents which is agreement with the results obtained in this study.

Some metals, such as Cu and Zn, are biologically essential to living organisms (Allan, 1997; Pinto et al., 2016). These essential elements are necessary in low amounts, and can be designated as micronutrients (Pinto et al., 2016). However, at higher concentrations, the same elements may become noxious to organisms (Allan, 1997; Jaishankar et al., 2014). In this study, Cu accumulation in sunflower roots was much higher than in shoots, especially for plants grown at 2.5 and 5 % of BC. Therefore, sunflower plants limited the translocation of Cu from roots to shoots, acting as metal excluder (Ali et al., 2013). With effect, sunflower grown at 2.5 and 5% of BC could be potentially used for phytostabilization purposes. Moreover, plants grown with 2.5% of BC and single inoculated with AMF and/or with PGPR/AMF consortia had a positive effect in Cu accumulation in roots. These bacterial strains exhibited several PGP traits (for instance siderophores synthesis), thus improving Cu accumulation in sunflower's tissues. In soil amended with 2.5 and 5% of BC it was observed an enhanced bioavailability of Cu-extractable forms, which are directly related to higher Cu content found in sunflowers' roots.

On the other hand, increasing BC rates induced an overall reduction of Zn content in shoots. However, Zn accumulation in roots (e.g. 85.23 – 333.92 mg Zn kg^{-1} plant dry weight) was generally lower than in shoots (e.g. 94.58 – 421.08 mg Zn kg^{-1} plant dry weight). Our study demonstrated that inoculated sunflower plants with AMF typically presented higher Zn levels in their tissues. With effect, Mani et al. (2015) showed that sunflower phytoremediation efficiency was improved with the use of microbial inoculants (e.g. PGPR and AMF), an organic amendment and nutrient supplementation. In another study, inoculation with the AMF *R. irregularis* increased Zn and Cd concentrations in sunflower shoots, but also enhanced phytoextraction of Cd (Hassan et al., 2013), which is agreement with the results of the present study. Indeed, AMF inoculation enhanced Zn accumulation in shoots at 5% and in roots at 2.5% of BC. Consequently, sunflower plants grown with 5% of BC and inoculated with the AMF *R. irregularis* can be potentially used for phytoextraction purposes. A similar trend was observed by Ali et al. (2017) that reports an increment in Zn content in sorghum shoots grown in soil amended with 1% of BC and inoculated with the strain *Streptomyces pactum* (Ali et al., 2017).

Through the analysis of Shannon diversity index (*H*), the present study showed that species diversity in the metal-contaminated soil decreased with microbial inoculation, particularly with AMF inoculation. On the other hand, rhizosphere bacterial diversity abundance, represented by *S* value that can symbolize the number of species found in a certain sample (Fromin et al., 2002), typically decreased between the first inoculation and the end of the experiment. This declining trend is in agreement to the results obtained by Marques et al. (2013) where the bacterial abundance in the rhizosphere samples underwent a reduction throughout the experiment. Evenness or equitability (*E*) index was calculated based on the Shannon's diversity, as previously described on section two. With effect, lower values for equitability index (near zero) expresses the dominance of one or few species in a certain sample, while higher values (close to one) point to complete evenness, where every species have an identical distribution (Morris et al., 2014; Pielou, 1975). In the current study, the equitability index varied between 0.904 and 0.987, showing a nearly equal species distribution among the different rhizosphere samples. The Simpson's Index of diversity, *D,* represents the probability of two individuals randomly selected from a certain sample to belong different categories (for example species) (Simpson, 1949). Values close to one represents a greater diversity in the rhizospheric soil studied in the present study. With effect, Shannon and Simpson's diversity indexes must have concordant results. It was observed a reduction in the number of bands from the beginning to the end of the experiment (e.g. initial and final number of bands for C_0 samples was 25 and 20 bands, respectively) in non-inoculated soils (C). On the other hand, the rhizospheric samples of Mix treatments were apparently able to sustain bacterial abundance, for instance, initial and final number of bands for Mix_0 samples was 24.

The current study aimed to evaluate how BC traits can influence rhizospheric bacterial communities and its' connection to plant related-aspects such as plant growth promotion (Kolton et al., 2011). Bacterial abundance found in BC-amended soils can be related not only to BC properties but also to soil native traits (e.g. texture) (Gul et al., 2015). According to Kolton et al. (2017) soil amendment with BC promoted alterations at low taxonomic levels in rhizospheric bacterial communities.

In summary, the present results indicate that not only bacterial communities differs throughout the experiment but is also dependent on the inoculation treatment applied to the soil. Particularly, soil inoculation with AMF promoted greater alterations in bacterial communities, for instance in terms of species abundance, which is in agreement with a preceding study (Moreira et al., 2016a). These alterations suggested that this inoculant could indirectly influence parameters associated to sunflowers' growth (e.g. the enhanced Zn accumulation verified for the plants cultivated at 2.5% of BC and inoculated with AMF). Important to highlight also that microbial inoculation, in general, did not induce higher bacterial diversity.

5. Conclusions/ Future work

This is one of the few works that studied the combined action of BC and bioinoculants for assisted-phytoremediation purposes. The current experiment can contribute for the restoration of metal-contaminated sites, characterized by the recovery of their ecosystem functionalities, along with the implementation of phytomanagement approaches at these locations. Overall, the results obtained in this study allowed the following conclusions:

1) BC amendment had a deleterious effect on sunflowers' biomass and height, while bioinoculation was capable to mitigate the negative influence of BC addition. Furthermore, mixed inoculation appeared to be the most beneficial inoculant, when compared to the remaining microbial treatments applied during the experiment. These results demonstrated the need to better realize how rhizobacteria and AMF consortia can improve plant growth and survival when exposed to stress conditions.

2) BC levels had different outcomes on Cu and Zn levels accumulated in sunflowers' roots and shoots at the end of the experiment. For instance, 5% of BC addition and inoculation with the AMF *R. irregularis* potentiated higher Zn accumulation in shoots, therefore showing sunflower's capacity to be used hereafter for phytoextraction approaches. On the other hand, amendments with 2.5 and 5% of BC and single inoculation with the strain *P. reactans* EDP28 or co-inoculated with the AMF promoted a higher Cu content in sunflower' roots. However, plants amended with the lowest amount of BC showed symptoms of nutrient deficiency, especially in sunflower leaves. These results showed that combining different amounts of BC and bioinoculants could provide different phytoremediation approaches, such as phytostabilization and/or phytoextraction.

3) Soil microbial community profiles varied along the experiment not only due to microbial inoculation but also to BC addition, particularly in terms of bacterial abundance. Overall, the plants inoculated with AMF showed lower bacterial diversity throughout the experiment, while the mixed inoculation tended to promote the maintenance of bacterial species found in the beginning of the experiment. BC addition at 5 and 10% enhanced

bacterial diversity, especially in non-inoculated plants, which is directly related to the number of bands recorded in DGGE profiles.

In a near future, it would be interesting to estimate the colonization rate of the AMF used in the present experiment. In addition, Cd levels in shoots and roots should be determined by FAAS. Similar factorial designs can be carried out with different types of BC differing for instance in application rate, pyrolysis temperature, pH and granulometry and with sunflower or with other crop with economic value and phytoremediation potential (e.g. wheat, maize). More PGPR and AMF consortia can be tested as phytoremediation assistants.

6. References

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