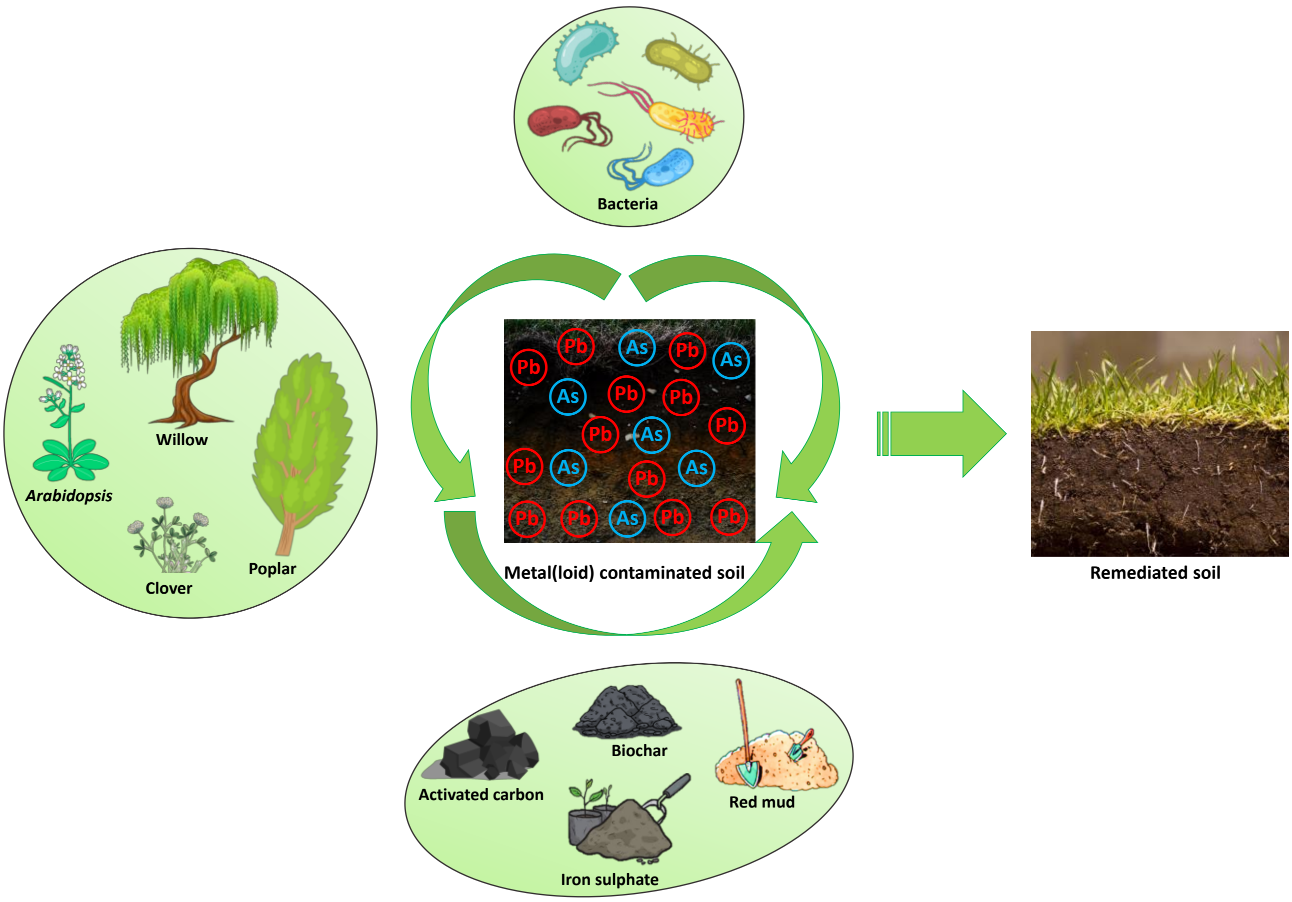
UNIVERSITY OF MOLISE

Department of Biosciences and Territory

DOCTORAL THESIS

Assisted phytoremediation of metal(loid) contaminated soils



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Assisted phytoremediation of metal(loid) contaminated soils

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Dedication

To mum, dad and Luca.

To determination.

Assisted phytoremediation of metal(loid) contaminated soils

Abstract

Metal(loid) contamination of soils is a global environmental problem with serious consequences on human, animal, plant and ecosystem health. In the course of the years, various technologies have been used to treat and remediate metal(loid) polluted sites. Assisted phytoremediation strategy for contaminated soils has attracted increasing attention since it offers environmental protection at low cost, and it can be applied to a large area. However, the efficiency of assisted phytoremediation of metal(loid) contaminated soils depends on environmental features (*e.g.*, type, pH and nutrient content of soil), contaminant characteristics, amendment types, microbial activity, plant species, and interactions between all these aspects.

Thus, the main objectives of thesis, related to applied and basic research, were to understand: (i) how different factors influence the effectiveness and the potential of assisted phytoremediation approach, and (ii) the mechanisms involved in the interactions between plants, amendments, microorganisms, and contaminated soil in assisted phytoremediation process.

To accomplish the objectives of the applied research, two experiments were carried out with the goal of providing insights for use of alternative methods for assisted phytoremediation of metal(loid) contaminated soils.

The first experiment was conducted using three different *Salicaceae* species and two diverse strategies of the same soil amendment. This experiment gave an outline about the influences of plant species and amendment application procedure on the assisted phytoremediation of a soil heavily polluted by only one metalloid (arsenic). The second experiment was focussed on the phytoremediation ability of a clover species and the need to use a combination of different amendments for the treatment of a multicontaminated soil. On the base of metal(loid) characteristics and soil physicochemical properties, experiment data showed the importance to choose a specific plant species and a correct combination of amendments (both in regarding to the amendment type and application rate).

In order to understand molecular mechanisms underlying plant tolerance to metal(loid) stress, plant-soil microorganism interactions and amendment effects, investigations were carried out by using the *Arabidopsis thaliana* model plant grown on a polluted soil added with amendment and bacteria. An in-depth bioinformatics-assisted proteomics analysis was also used for deciphering the biological processes which allowed the better growth of *Arabidopsis* plants, in the soil with amendment/bacteria combination.

Taking together, data of this thesis provided important suggestions on how to use different combinations of amendments and microorganisms in assisted phytoremediation strategies in order to efficiently treat soils contaminated by inorganic pollutants. Furthermore, results obtained on the *A. thaliana* model plant contributed with new information in broadening the knowledge on the complex interactions occurring among contaminants, plants, soil amendments, and microorganisms in areas polluted by high concentrations of metal(loid)s. Such information might be applied in the selection and management of plant species, amendment types, and soil microbial communities to ameliorate plant-amendment-microbe synergy, increase plant growth and tolerance to biotic and abiotic stresses and, consequently, enhance the efficiency of phytoremediation programs to restore metal(loid) contaminated lands.

Contents

List of publications

This thesis is based on the work contained in the following papers:

I. **Assisted phytoremediation of a former mine soil using biochar and iron sulphate: effects on As soil immobilization and accumulation in three *Salicaceae* species**
Melissa Simiele, Manhattan Lebrun, Florie Miard, Dalila Trupiano, Philippe Poupart, Olivier Forestier, Gabriella S. Scippa, Sylvain Bourgerie, Domenico Morabito.
(2020) Science of the Total Environment, 710:136203.
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II. **Evaluation of different amendment combinations associated with *Trifolium repens* to stabilize Pb and As in a mine-contaminated soil**
Melissa Simiele, Manhattan Lebrun, Giorgia Del Cioppo, Gabriella S. Scippa, Dalila Trupiano, Sylvain Bourgerie, Domenico Morabito.
(2020) Water, Air and Soil Pollution, 231:539.
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III. **In-depth study to decipher mechanisms underlying *Arabidopsis thaliana* tolerance to metal(loid) soil contamination in association with biochar and/or bacteria**
Melissa Simiele, Gabriella Sferra, Manhattan Lebrun, Giovanni Renzone, Sylvain Bourgerie, Gabriella S. Scippa, Domenico Morabito, Andrea Scaloni, Dalila Trupiano.
(2020) Environmental and Experimental Botany.
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1 Introduction

Trace metals and metalloids (referred as metal(loid)s hereafter) are biologically essential for organisms to maintain normal life activities (Tang et al., 2019). However, excessive metal(loid) accumulation is harmful to animal life, human health, and plants. High concentrations of metal(loid)s are accumulated in soil through many pathways, including mining, smelting and associated activities for human purposes (Kříbek et al., 2019). Among the most common metal(loid)s found at contaminated sites, arsenic (As), lead (Pb), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), and zinc (Zn) are quite abundant (Wuana and Okieimen, 2011). Contamination of soil by metal(loid)s is of great concern due to their toxicity, non-biodegradability, and accumulative behaviour (Fajardo et al., 2019). Consequently, the remediation of metal(loid) polluted sites became mandatory to reduce the associated risks. Several technologies exist to remediate metal(loid) contaminated soils. Remediation approaches can be classified into three main categories: (i) physical processes, (ii) chemical processes, and (iii) biological processes. The physical remediation consists of soil replacement method and thermal desorption (Qayyum et al., 2020); chemical processes destroy, fix or concentrate contaminants by using one or more types of chemical reaction (Ye et al., 2017), whereas the biological remediation uses living organisms (Yao et al., 2012). Physicochemical strategies to remediate contaminated soils have been used for a long time, but these methods have many limitations. They are generally expensive and may damage, often irreversibly, native soil flora and fauna, and induce secondary pollutions stemming from the chemical agents added in the processes (Xu et al., 2019). In order to overcome these problems, biological methods are an attractive alternative to conventional remediation techniques. The biological remediation includes phytoremediation (plant use), bioremediation (microorganism use), and the combining remediation (combined use of plants and microorganisms) (Kumar and Gunasundari, 2018). These biological strategies are environment-friendly green technologies characterized by simplicity and cost-effectiveness (Landa-Acuña et al., 2020). In recent years, a new biological remediation known as "assisted phytoremediation" is proving to be a promising option to manage lands contaminated with metal(loid)s (Yang et al., 2020). Assisted phytoremediation can be defined as a remediation strategy that uses vegetation, associated microbiota and soil amendments to remove, contain, or render environmental contaminants harmless (Wuana and Okieimen, 2011). This new promising remediation approach that involves plant-microorganism-amendment combination is not yet fully explored for the use on contaminated soils. Furthermore, still completely unknown are the mechanisms involved in the interaction between plants, microorganisms and amendments and underlying the response of plants to metal(loid)s (Hasan et al., 2019; Yang et al., 2020). Elucidation of how microorganisms and different amendment types affect plant growth and development in contaminated soils, and a better understanding of the tolerance mechanisms by which plants respond to metal(loid) stress is of great interest for plant biologists, and for the scientific community involved in the development of technologies for soil remediation (Wuana and Okieimen, 2011).

1.1 Metal(loid) contaminated soils

Soil is considered a dynamic, complex, and full biodiversity habitat that provides many benefits to humans and ecosystem survival (Yao et al., 2012). Nonetheless, with industrialization, urbanization and intensified agriculture, soils are increasingly contaminated by potentially toxic elements (Yang et al., 2020). Although soil may be polluted by different organic and inorganic contaminants, metal(loid)s seem to be more serious than other contaminations (Guo et al., 2019). Metal(loid)s are ubiquitous inorganic elements in the earth's environment and can result from both natural and anthropogenic activities (He et al., 2015) (Fig. 1). Indeed, metal(loid)s are natural constituents of soil and, some of them are involved in physiological functions of living beings (*e.g.*, humans, plants, animals and other organisms) (Begum et al., 2019).

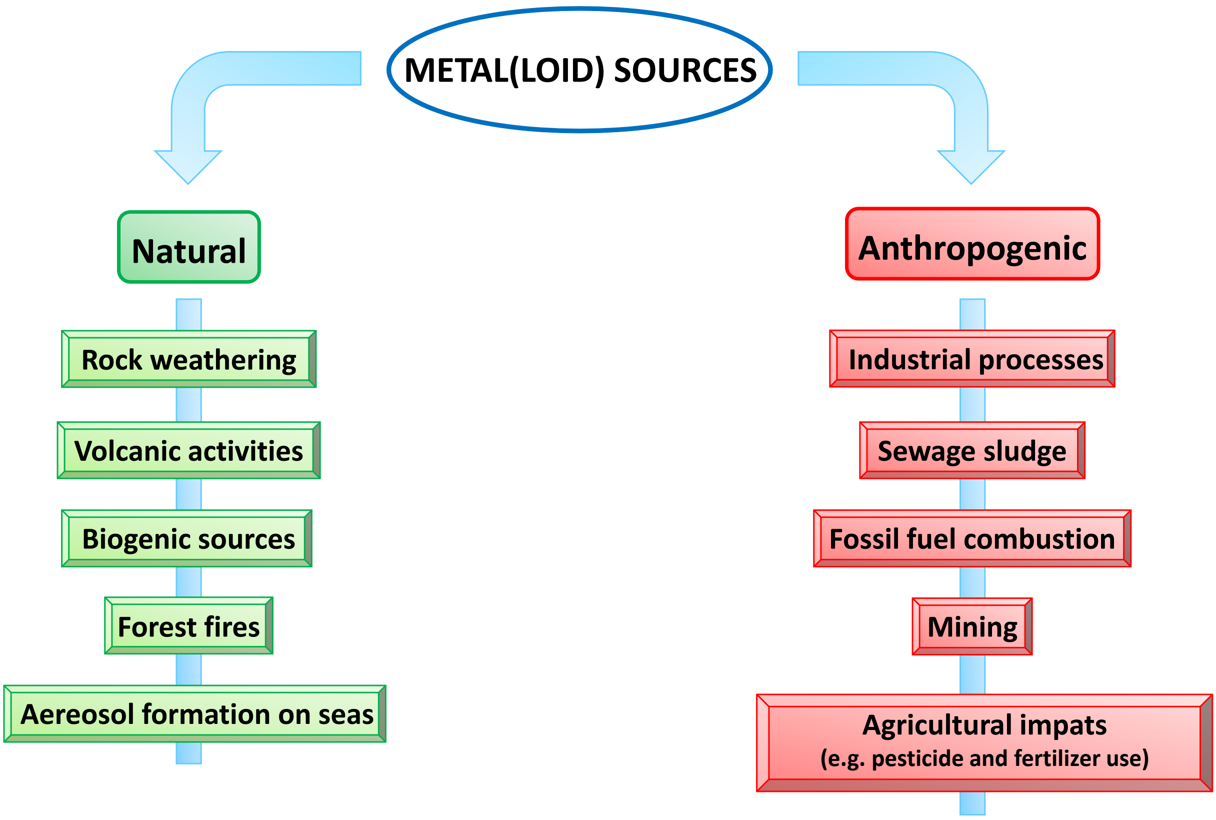

Fig. 1 ***Sources of metal(loid)s***

As listed in Table 1, metal(loid)s can be classified as (i) nutritionally essential, (ii) nonessential with a possible beneficial effect, and (iii) nonessential with no beneficial effects (Goyer et al., 2004). The nonessential elements are potentially toxic even at low concentrations, while the essential ones can exert harmful impacts when present at high concentrations (Begum et al., 2019). The intensification of various agricultural and industrial activities, for human purposes, including fossil fuel combustion, agricultural use of fertilizers and pesticides, mining waste, and landfill leaching have led to a significant increase of soil contamination by metal(loid)s (Qayyum et al., 2020). The most common metal(loid)s present in the environment are arsenic (As), lead (Pb), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), and zinc (Zn) (He et al., 2015).

Nutritionally essential metal(loid)s	**Metal(loid)s with possible beneficial effects**	**Metal(loid)s with no known beneficial effects**
Cobalt	Boron	Aluminum
Chromium (III)	Nickel	Antimony
Copper	Silicon	Arsenic
Iron	Vanadium	Barium
Manganese		Beryllium
Molybdenum		Cadmium
Selenium		Lead
Zinc		Mercury
		Silver
		Strontium
		Thallium

Table 1. ***Classification of metal(loid)s based on the health impact characteristics*** (Goyer et al., 2004)

Metal(loid) are carcinogenic and mutagenic, and they would arouse amplification effects through the food chain in organisms, causing great threats to human health, and natural ecosystems (Ye et al., 2017). Risks and hazards to humans and ecosystems posed by metal(loid) soil contamination are caused through (i) direct ingestion or contact with polluted soil, (ii) the food chain (soil-plant-human or soil-plant-animal-human), (iii) drinking of contaminated groundwater, (iv) reduction in food quality via phytotoxicity, (v) reduction in land usability for agricultural production causing food insecurity, and (vi) land tenure problems (Kim et al., 2015; Wuana and Okieimen, 2011). Metal(loid)s are more dangerous than other pollutants because most of them do not undergo microbial or chemical degradation, and their total concentration in soil persists for a long time after their introduction (Wuana and Okieimen, 2011). Indeed, Kabata-Pendias (2011) estimated that residence times were

75-380 years for Cd, 500-1000 years for Hg, and 1000-3000 years for Pb, Cu, and Zn. Worldwide, soils of more than 10 million sites have been reported as being polluted, and more than 50% of these sites resulted contaminated by metal(loid)s (EPMC, 2015). In 2007, Kabata-Pendias and Mukherjee compiled a report on the highest concentrations of some metal(loid)s measured in soils contaminated from industrial sources in selected countries. As reported by these authors, China resulted to have the highest As and Hg concentrations (2500 and 100 $mg \cdot kg^{-1}$), the United States the highest Cd, Pb and Zn concentrations (1500, 13000 and 80000 $mg \cdot kg^{-1}$, respectively), while Canada the highest Cu concentration (3700 $mg \cdot kg^{-1}$). The European Commission (2013) reported that in European countries, fertilizers and the large volumes of municipal and industrial wastes have caused significant soil contamination by metal(loid)s. In 38 European countries, more than 2.5 million sites are potentially contaminated, while 342,000 sites have been identified as contaminated sites (Wcisło et al., 2016).

1.2 Metal(loid) effects on soil and microorganisms

The term “soil health” or “soil quality” is used to express the status of the soil functional ability in the ecosystem, as indicated by its physical, chemical and biological properties (Doran and Zeiss, 2000). There are wide discrepancies in defining metal(loid) critical limits in soil among different countries and these limits also differ according to the type of soil (urban, agricultural or industrial soil) (Bakshi and Banik, 2018). However, whatever is the critical limit, metal(loid) contamination above threshold values can destroy the soil natural ability to perform ecosystem services, a change which can be irreversible (Cheng et al., 2020). Indeed, metal(loid)s affect chemical, biochemical and microbiological soil characteristics. Metal(loid) contamination can cause soil acidification and subsequently affects other soil properties (Yu et al., 2006). It has been observed that soil pH decreases with the input of metal(loid)s in the order of Cu = Pb > Cd = Ni = Zn (Basta and Tabatabai, 1992). By interactions with metal(loid)s, soil organic content has been found to be modified, in terms of decreased turnover (Quenea et al., 2009). Furthermore, metal(loid) contamination modifies the quality of soil organic matter, especially by misbalancing its composition (Martinez-Toledo et al., 2016). Indeed, organic matter is characterized by negatively charged sites which on the one side may be involved in complexation, precipitation and adsorption reactions of metal(loid) cations, while on the other side may be involved in desorption and mobilization of metal(loid) anions (*e.g.*, arsenic anions) in soil (Bakshi and Banik, 2018). In addition, metal(loid)s inhibit soil enzymatic activity damaging soil organic matter mineralization and nutrient cycling (Martinez-Toledo et al., 2016). Metal(loid)s inhibit soil enzyme activity throughout the (i) masking of catalytically active groups, (ii) denaturation of enzyme conformation, and (iii) competition with enzyme-substrate (Sun et al., 2007). Enzymatic activities are also indirectly affected by the modifications of the soil microbial community induced by metal(loid)s (Shun-hong et al., 2009).

It has been recently reported that high concentrations of metal(loid)s reduce the size and the diversity of the microbial community in the soil resulting in a loss of soil functions (Bakshi et al., 2018). Metal(loid)s have also negative effects on soil microbial activity affecting carbon and nitrogen metabolism (Chen et al., 2014; Nwuche and Ugoji, 2008). However, although the number and diversity of species in the soil microbial community are affected by metal(loid) accumulation, evidences showed that the increase of metal(loid) concentrations also enhances the development of metal(loid)-resistant microorganisms (Xie et al., 2014). Microbial communities have a different tolerance to metal(loid) contamination and the degree of this tolerance varies in the order of fungi > bacteria > actinomycetes (Hiroki, 1992). Indeed, microorganisms in polluted soils may develop specific mechanisms to overcome the presence of metal(loid)s. As shown in Fig. 2, the main mechanisms to overcome metal(loid) toxicity include extracellular sequestration, intracellular sequestration, active transport of metal(loid)s, and enzymatic detoxification (Yin et al., 2019).

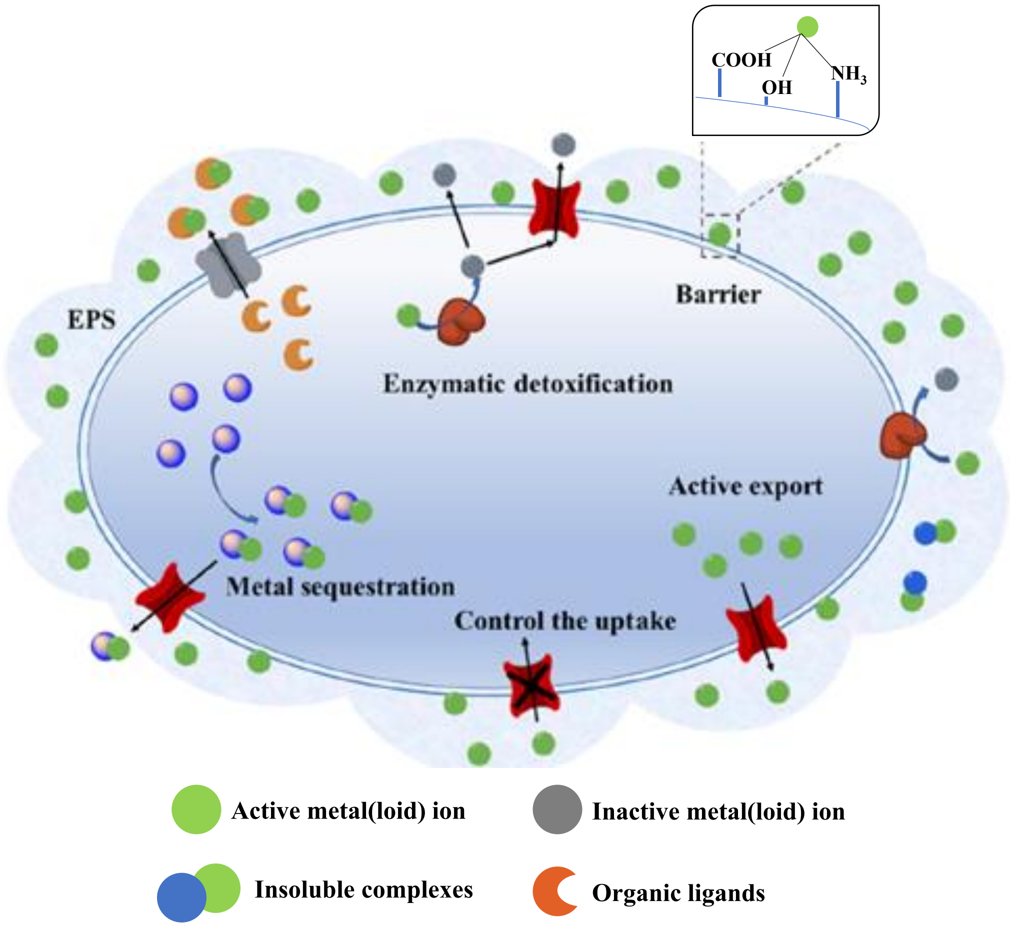

Fig. 2 ***Some detoxification mechanisms of microorganisms towards metal(loid)s***
EPS: extracellular polymeric substances (adapted from Yin et al., 2019)

Extracellular sequestration mechanisms avoid metal(loid) entrancing into the intracellular environment. In this case, metal(loid) ions can be bonded by various biological structures such as extracellular polymeric substances (EPS), siderophores, and glutathione (Costa et al., 2018; Yin et al., 2019). The first component of microbial cell that is encountered with metal(loid) ions is the cell wall, which is a very important defence system against metal(loid) toxicity. The great number of cationic and anionic functional groups on cell wall, such as hydroxyl, amine, carboxyl and phosphate groups, are able to avoid the entrance of metal(loid) ions into the intracellular environment by chelating them (Nanda et al., 2019). EPS include nucleosides, lipids, proteins, and complex carbohydrates which have carboxylic, hydroxyl, amino, sulfhydryl and phosphate functional groups able to sequestrate metal(loid) ions (Shi et al., 2017).

Siderophores are organic ligands excreted by bacteria and fungi with the ability to accumulate ferric ions helping microorganisms to survive under iron-deficient conditions (Ghosh et al., 2020). Besides, siderophores can also bind metal(loid)s (*e.g.*, Cu, Ni and Zn) protecting microorganisms from their toxicity (Sharma et al., 2018). Glutathione secreted by microorganisms also has great ability to bind and adsorbed metal(loid) ions to form complexes and cannot enter living cells (Khullar and Reddy, 2016).

Once metal(loid) ions enter microbial cells, different molecules within the cytoplasm can sequestrate these ions and prevent them from reaching toxic levels. Therefore, sensitive cellular components and organelles can be protected from the exposure to metal(loid) toxicity (Yang et al., 2020). Many microorganisms can transform active metal(loid) ions to insoluble metal(loid) precipitates with the help of sulphides, cytosolic polyphosphates and cysteine rich proteins like metallothioneins (Blindauer et al., 2008).

Active transport of metal(loid)s away from the intracellular environment is another process to efficiently regulate intracellular concentrations of metal(loid) ions in microorganisms (Yin et al., 2019). It has been reported that specific resistance genes on chromosome or plasmid regulate the expression of metal(loid) ion transporters which depends on bacterial species and metal(loid) ions (Wang et al., 2008). Microbial membrane transporters are able to control the uptake and exclusion of metal(loid)s into and from intracellular environment. There are numerous metal(loid) exporting proteins widespread in the cell membrane to achieve the efflux of metal(loid) such as P-type efflux ATPase, proton-cation antiporters, ABC transporters and cation diffusion facilitator (Lerebours et al., 2016).

Another intracellular defence pathway microorganisms use to cope with metal(loid) toxicity is biotransformation or chemical modification of metal(loid)s. Indeed, microorganisms possess detoxification enzymes able to change the redox state of metal(loid) ions converting them from a high toxic form to a less harmful one (Giovanella et al., 2016).

1.3 Metal(loid) effects on plants

Much evidence showed that some metal(loid)s are considered to be essential for plant growth, however, at high concentrations, metal(loid)s may be hazardous to plants, and their phytotoxicity increases with increasing metal(loid) concentrations (Ghani, 2010). For example, some metal(loid)s, like Cu and Zn, are important cofactors and enzyme activators, but others, such as As, Cd and Hg, are extremely poisonous (Ackova, 2018). The decline in plant growth as well as leaf necrosis, decrease in the rate of seed germination, and turgor loss are most common visual evidence of metal(loid) stress (Bakshi et al., 2018). However, many evidences have been reported that high concentrations of metal(loid)s in the soil can cause plant oxidative stress (Siddiqui et al., 2011), damage cell structure by substituting nutrients (Gulz et al., 2005), affect enzyme activity (Yusuf et al., 2012), and hamper photosynthetic reactions (Sethy and Ghosh, 2013). Metal(loid)s can affect physiological and biochemical processes in plants and their toxicity varies with plant and metal(loid) species, metal(loid) concentration and chemical form (Dubey et al., 2018).

Zn is an essential element required by plant for its normal growth and development (Ghori et al., 2019). Zn plays an important role in the biosynthesis of enzymes, proteins and important plant hormones like auxins (Ackova, 2018). However, when Zn is present at high concentrations, it causes leaf chlorosis and induce decrease in plant growth, root length and plant height, alteration in metabolism processes, and induction of oxidative damage (Chen et al., 2017). Moreover, Zn toxicity results in inhibition of photosystems I and II influencing photosynthesis and negatively affects the overall synthesis of ATP; nevertheless, these effects are reversible although they may become permanent if the stress persists (Ghori et al., 2019).

Plants facing Cd toxicity usually have retarded growth and rolled chlorotic leaves (Li et al., 2016). Cd accumulation affects the photosynthesis throughout the many different ways: (i) inhibition of enzyme activity of Fe (III) reductase that leads to Fe (II) deficiency, (ii) reduction of chlorophyll synthesis, and (ii) inhibition of enzymes involved in CO_2 fixation (Huang et al., 2017). Cd toxicity leads to the inactivation of enzymes involved in oxidative stress regulation such as superoxide dismutase, peroxidase, catalase and ascorbate peroxidase (Tauqeer et al., 2016). Cd also interferes with uptake, transport and use of Ca, K, P, Mg and water and reduces the translocation and absorption of nitrate by inhibiting nitrate reductase (Tran and Popova, 2013). Water balance disturbance has also been noticed which is caused by disturbed plasma membrane integrity during lipid peroxidation (Nagajyoti et al., 2010).

Pb is counted among the major metal(loid)s that contaminate the soil, resulting from smelting and mining activities (Ashraf et al., 2015). The symptoms of Pb toxicity in plants are reduced growth, chlorosis and reduced root lengths (Ghori et al., 2019). Once entered into the cell, Pb causes cell membrane permeability changes, inhibition of various enzymes containing sulfhydryl group, reduction in water content and alteration in mineral nutrition (Dubey et al., 2018). Pb toxicity disrupts chloroplast ultrastructure and blocks synthesis of essential pigments (*e.g.*, chlorophyll and carotenoids) with negative consequences on photosynthetic pathways. Moreover, it blocks the Calvin cycle and electron transport chain (Sharma and Dubey, 2005).

As is easily absorbed by plant root cells, and once entered into the cell As (V) can be converted to the more toxic forms, As (III), disrupting plant metabolism (Liu et al., 2012). Phosphate-dependent metabolism can be interrupted by As (V) being chemically similar to phosphates, and it can also move across cellular membranes by phosphate transporters, causing imbalances in phosphate supply. During phosphorylation reactions, As (V) can compete with phosphate and uncouples the oxidative phosphorylation by displacing phosphate during ATP synthesis (Finnegan and Chen, 2012). As (III) potentially inactivates the enzymes containing cysteine residues or dithiol cofactors (Meharg and Hartley-Whitaker, 2002).

However, although high concentrations of metal(loid)s cause harmful effects on cellular and physiological processes in plants, there are many species that have evolved several complex strategies to cope with metal(loid) stress (Wang et al., 2008).

Plants utilize a series of defence mechanisms to control metal(loid) uptake, accumulation and translocation, and these defence strategies are distinguished in tolerant and detoxification mechanisms (Mecwan Neha et al., 2018). Based on the type of defence mechanism employed, plants that can grow in the presence of metal(loid)s are categorized in excluders and accumulators/hyperaccumulators (Peer et al., 2006) (Fig. 3).

Excluders are plants able to tolerate high metal(loid) concentrations by preventing their entrance and accumulation in plant cells. To hinder the metal(loid) entrance into root cells, one commonly used strategy consists in the entrapment of metal(loid)s in the soil by binding them to exudates or to cell walls (Rascio and Navariizzo, 2011). On the other hand, in these plants, the metal(loid) accumulation is avoided by energy dependent efflux pumps (Leitenmaier and Küpper, 2013). Accumulators and hyperaccumulators plants take metal(loid)s up actively from soil and concentrate them in root, shoot and leaf tissues to levels far exceeding that in the soil without any evidence of physiological stress (Castaňares and Lojka, 2020). Indeed, hyperaccumulator plants can retain metal(loid)s in their organs at concentrations 100-1000-fold higher than those found in non-hyperaccumulating species (Rascio and Navariizzo, 2011).

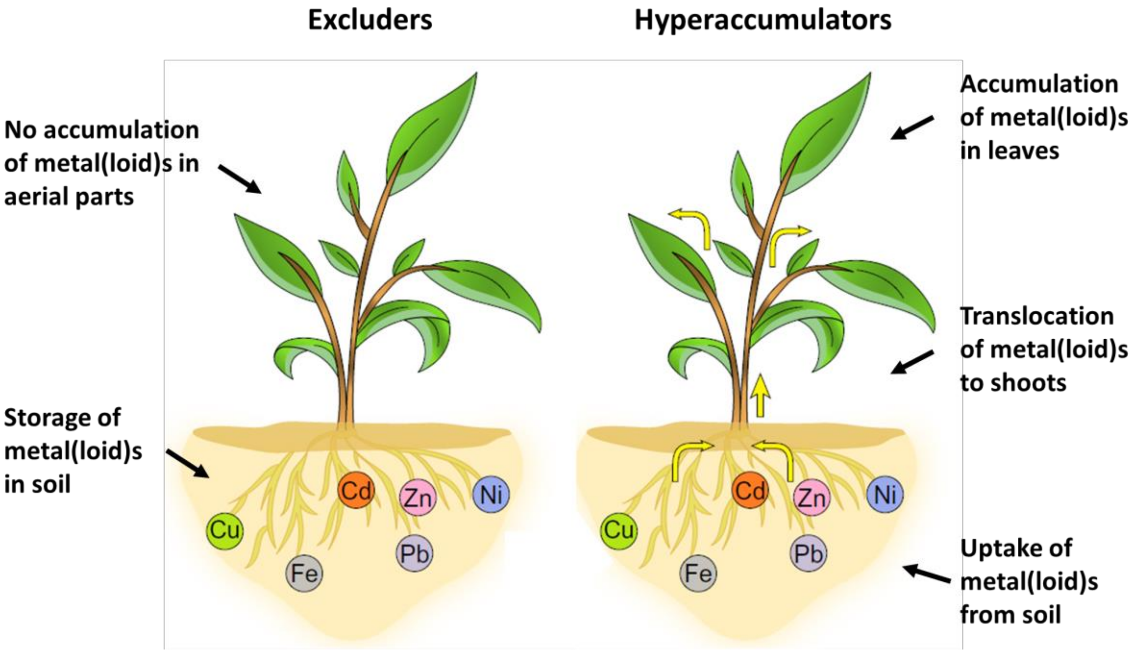

Fig. 3 ***Main differences between excluder and hyperaccumulator plants*** (adapted from Ghori et al., 2016)

Most of the metal(loid)s that enter hyperaccumulator plants are detoxified by complexation with amino acids, organic acids or metal-binding peptides and/or sequestered into vacuoles (Leitenmaier and Küpper, 2013). A further defence mechanism generally adopted by metal(loid)-exposed plants is enhancement of cell antioxidant system which counteracts oxidative stress (Peer et al., 2006).

Some of these mechanisms are constitutive while others are adaptive and are activated only as the stress due to the high level of metal(loid)s is encountered (Dubey et al., 2018). Usually, plants have more than one mechanism to overcome different metal(loid) stresses that act at different sites within the plant (Fryzova et al., 2018). These mechanisms allow plant to bind specific metal(loid) ions, sequester and exclude them into compartments and preventing their entrance in other cellular organelles (Ghori et al., 2019). When a plant grows in polluted soils, it reduces metal(loid) ion uptake via roots as first line of defence. Indeed, plants can restrict metal(loid) uptake by various root exudates (*e.g.*, malic and citric acid). Root exudates have been reported to often bind metal(loid) ions or attach them to cell wall (Pinto et al., 2008). Pectin is one of the essential components of cell walls that is required for sequestering metal(loid)s (Krzesłowska, 2011). The cell wall also deposits callose that does not allow metal(loid)s to enter into intracellular environment (Ghori et al., 2019). Moreover, the cell wall is rich in compounds such as carbohydrates, amino acids, phenolics and proteins which offer functional groups (–COOH, –SH, –OH) able to bind metal(loid) cations (Ghori et al., 2019). Another mechanism to reduce metal(loid) build up in plants is the use of efflux transporters which are able to detoxify metal(loid)s by excreting them outside the cell (Singh et al., 2015). Zhao et al. (2010) demonstrated that an aquaporin, involved in As (III) uptake, was also involved in its efflux. In similar way, Lee et al. (2007) showed that some ATPases are transmembrane metal(loid)-transporting proteins that play an important role in Cu, Pb and Zn efflux.

If metal(loid)s succeed in entering cells, plants are able to activate other defence mechanisms to deal with such stress. Metal(loid)s are usually removed from the cytosol and enclosed in vacuoles which are the major sites of metal(loid) ion storage inside the cell (Dubey et al., 2018). Metal(loid)s can be chelated by metal-binding molecules (*e.g.*, glutathione, phytochelatins and metallothioneins) in the cytosol upon their entry. Glutathione and phytochelatins can form complexes with metal(loid)s that are transported and then compartmentalized in the vacuoles where a complex cascade of metal(loid) detoxification initiates (Hossain et al., 2012) Two proton pumps, namely vacuolar proton-ATPase and vacuolar proton pyrophosphatase, are responsible for pumping metal(loid)s inside the vacuole where they are sequestrated and detoxified by binding to vacuolar peptides and other molecules (Dubey et al., 2018). Plants also use antioxidant system as a tool for resistance against metal(loid) toxicity (Emamverdian et al., 2015). Antioxidant defence system includes antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, catalase, glutathione reductase, glutathione S-transferase, and guaiacol peroxidase, and also low molecular weight antioxidants such as glutathione, ascorbate, and carotenoids which provide significant contribution in metal(loid) detoxification (Das and Roychoudhury, 2014).

1.4 Strategies for the remediation of metal(loid) contaminated soils

The remediation of metal(loid) soil pollution is not easy process but many remediation strategies have been proposed for metal(loid) contaminated soils. Yao et al. (2012) classified the remediation techniques for soil contamination in (i) physical, (ii) chemical, and (iii) biological approaches (Fig. 4). Physical remediation involves soil replacement and thermal desorption (Bakshi and Banik, 2018). These remediation methods are simple techniques that use mobile devices and give the possibility to reuse the remedied soil (Qayyum et al., 2020). Chemical technologies include chemical leaching, chemical fixation and electrokinetic remediation (Qayyum et al., 2020). Of these methods, chemical leaching is considered environmentally friendly and cost-effective (Alam et al., 2001). However, physicochemical technologies have many limitations: (i) require expensive devices, (ii) sometimes, their remediation time are long, (iii) need an intensive labor, and (iv) have negative environmental effects (Aresta et al., 2008; Bolan and Duraisamy, 2003; Fu, 2008; Guo et al., 2019; Zhou et al., 2004). On the contrary, biological strategies are cost-effective, environmentally friendly and have a wide public support (Hasan et al., 2019). Indeed, these technologies use solar energy and ensure that the soil properties are conserved (Kang et al., 2016). Biological remediation approaches include bioremediation, phytoremediation, lower animal remediation, and their combination (Bakshi and Banik, 2018). In this thesis, attention is given to the technologies of bioremediation and phytoremediation.

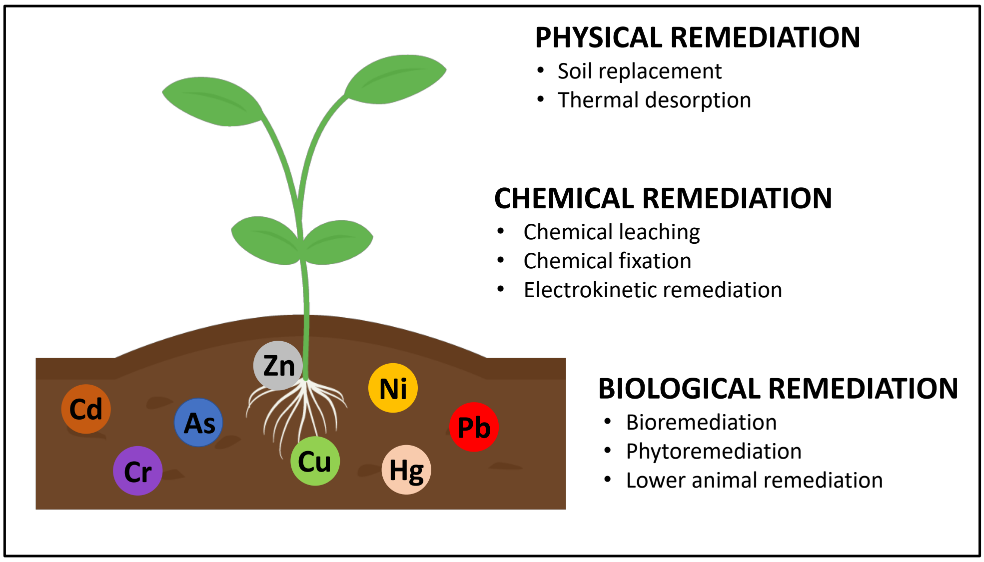

Fig. 4 ***Remediation strategies for metal(loid) contaminated soils***

1.4.1 Bioremediation of metal(loid) contaminated soils by microorganisms

Bioremediation is defined as the process that uses various biological agents, primarily microorganisms, to remediate polluted soils (Sharma, 2012). The United States Environmental Protection Agency (U.S. EPA) has defined bioremediation agents as microbiological cultures, enzyme and nutrient additives that significantly increase the mitigation of the negative effect of various pollutants (Sharma, 2012). Bioleaching, bioreactor, bioventing, composting, land farming, bioaugmentation, and biostimulation are all examples of bioremediation technologies (Li et al., 2011). Various factors can affect bioremediation processes of metal(loid) polluted soils and are microbial populations, chemical factors regarding metal(loid) characteristics, and environmental factors (*e.g.*, pH, nutrients, soil type, temperature, and water content) (Yang et al., 2020). The various types of microorganisms used for the bioremediation of metal(loid) contaminated areas are bacteria (*Bacillus* *substilis*, *Desulfoviibrio desulfuricans, Pseudomonas putida*, etc.), fungi (*Aspergillus fumigates*, *Penicillium canescens*, etc.), algae (*Cladophora* spp. and *Spirullina* spp.), and yeast (*Candida utilis* and *Saccharomyces cerevisiae*) (Hassan et al., 2017).

The bioremediation is based on the ability of microorganisms to develop a resistance to metal(loid) by processes of metal(loid) sequestration, complexation, exclusion, and detoxification. Extracellular and intracellular materials bind metal(loid)s and prevent their entry in microbial cells and organelles (Wang et al., 2014). These substances together with siderophores secreted by microorganisms remove metal(loid) toxic effects and limit their bioavailability by forming complexes (Liu et al., 2017). Once entered into cells, metal(loid) ions can be compartmentalized and detoxified with different microbial defence pathways (Ahemad, 2014). Generally, the uptake capacities of microorganisms for metal(loid) ions range from 1 $mg \cdot g^{-1}$ to 500 $mg \cdot g^{-1}$ and change depending on the type of metal(loid) and microbial species (Yin et al., 2019).

In general, metal(loid) ions may be adsorbed on the negatively charged carboxylic, hydroxyl, and phosphoryl groups on the bacterial cell wall (Ahemad and Kibret, 2013). Wu et al. (2010) reported that peptidoglycan, phosphoryl and chitin are the primary binding molecules for metal(loid) complexation for the cells of gram-positive bacteria, gram-negative bacteria and fungi, respectively. Moreover, microorganisms have been widely used for the treatment of polluted soils, because metal(loid)-reducing microbes are able to convert metal(loid)s from toxic soluble forms into less toxic ones through enzymatic transformation which can be changed metal(loid) redox state (Rascio and Navariizzo, 2011). *Arthrobacter*, *Bacillus*, *Pseudomonas*, and *Saccharomyces* are a few examples of microorganisms known to conduct the oxidoreduction transformations (Gadd, 2000). Indeed, some bacteria like *Bacillus substilis* and *Pseudomonas putida* are effectively used for the conversion of toxic Cr (VI) into less toxic Cr (III) (Purwanti et al., 2017). *Desulfoviibrio desulfuricans* is a sulphate-reducing bacterium which indirectly changes sulphate into hydrogen sulphate that consequently interacts with Cd and Zn to produce insoluble form of them (Kumar and Gunasundari, 2018). An important role in restoring contaminated lands has also been found in arbuscular mycorrhizal fungi whose tolerance increases with long-term metal(loid) exposure (Peréz-de-Mora et al., 2005). Filamentous fungi, such as *Gibberella*, *Aureobasidium*, *Saccharomyces* and *Phellinus*, are resistant to metal(loid) ions and can absorb them in significant quantities (Liu et al., 2017).

1.4.2 Phytoremediation of metal(loid) contaminated soils

The use of plants to remove or neutralize contaminants in polluted sites is defined phytoremediation and is based on the response of many plant species to the presence of metal(loid)s in the soil (Vidali, 2001). Among phytoremediation technologies, there are several approaches used to manage metal(loid) polluted soils (Sharma, 2012). Phytoremediation can be conducted in many ways depending on plants ability to degrade, extract, contain, or immobilize contaminants from soil (Fiorentino et al., 2018). In the emerging technology of phytoremediation, that uses plants to remove contaminants from the environment or immobilize them in soil, it is possible to classify different types of techniques on the base of contaminant fate: (i) phytostabilization, (ii) phytoextraction, (iii) phytodegradation, (iv) rhizodegradation, and (v) phytovolatilization (Fiorentino et al., 2018) (Fig. 5). All of these technologies are based on the different mechanisms, described earlier in this thesis, that plants possess or have evolved to cope with metal(loid) stress (Dubey et al., 2018)

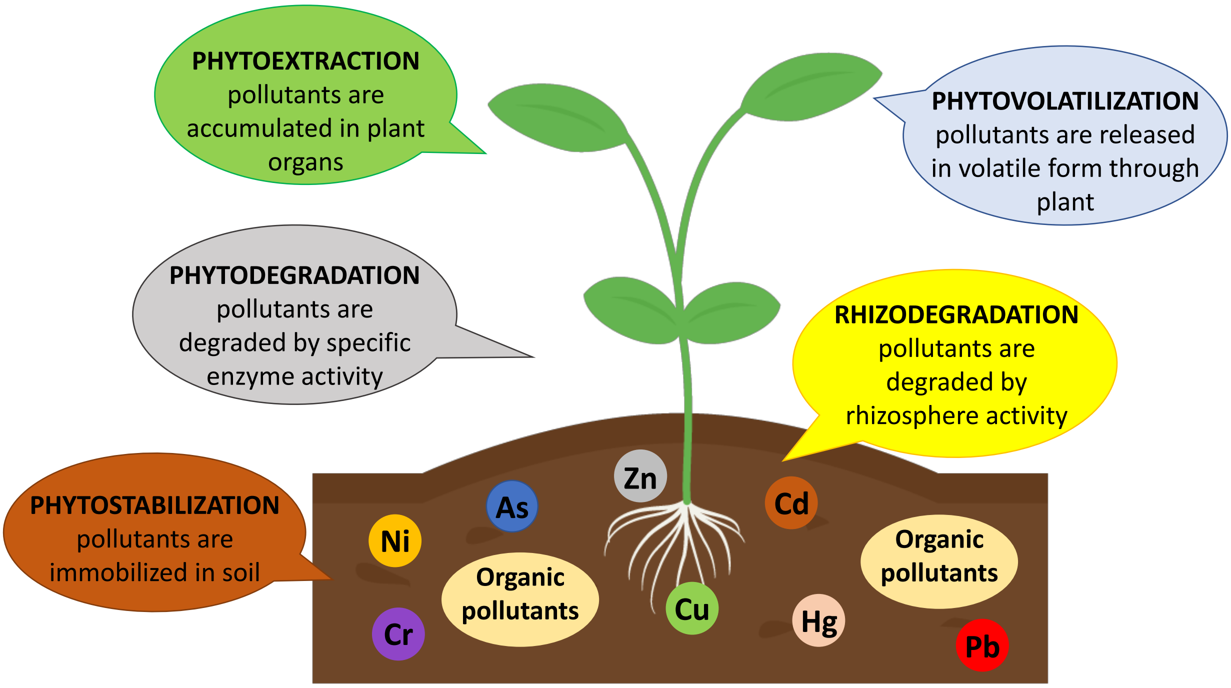

Fig. 5 ***Different types of phytoremediation strategy***

Phytostabilization is the use of plants to reduce bioavailability and mobility of contaminants in polluted soils and limit exposure to them (Sylvain et al., 2016). The reduction of metal(loid) availability and mobility is possible through compartmentalization and reduction/precipitation mechanisms in plant root layer (Mahar et al., 2016). These processes have also an important role of protecting groundwater from metal(loid) contamination (Palladino et al., 2018). Both herbaceous and woody plants can be used in phytostabilizing metal(loid)s (Singh et al., 2017). For example, various species of *Agrostis* and *Festuca* were used in phytostabilization of Cu, Zn, Pb contaminated soil from Europe, China and America (Pastor et al., 2015; Singh et al., 2017). Woody plants are able to fixed metal(loid)s on their roots or stabilize them into the soil and, moreover, these plants can reduce soil erosion (Singh et al., 2017). Different species of woody plants (*e.g.*, *Populus* spp. and *Quercus* spp.) were able to stabilize Cd, Cu, Pb and Zn in multi-contaminated metal(loid) soils (Domínguez et al., 2009; Hu et al., 2013).

Phytoextraction, also known as *phytoaccumulation*, is the process used by plants to accumulate pollutants into the roots and aboveground shoots or leaves (Ghori et al., 2016). Metal(loid) phytoextraction can significantly reduce the bioavailable fraction of contaminants to values below the risk thresholds. In phytoextraction methods, metal(loid) uptake can be significantly increased by selecting appropriate plant species (Fiorentino et al., 2018). The most suitable species for phytoextraction may have several of the following characteristics (Alkorta et al., 2004): (i) tolerance to high metal(loid) concentration, (ii) metal(loid) accumulation in easily harvestable organs, (iii) fast-growing biomass and high biomass yield, (iv) high root growth, (v) easy cropping management, and (vi) biomass useful for energy production or green chemistry. Currently, more than 400 plant species belonging to 45 families have been identified as hyperaccumulators (Khalid et al., 2017). These plants are mainly included in families like *Asteraceae*, *Brassicaceae, Caryophyllaceae*, *Euphorbiaceae, Fabaceae, Lamiaceae, Poaceae,* and *Violaceae* (Singh et al., 2017). Some species of *Brassicaceae* have the ability to accumulate metal(loid)s in their tissues. Cd, Pb, Ni and Zn are the most common metal(loid)s that *Brassicaceae* extract from polluted soils (McGrath et al., 2001). It is found that *Brassica juncea* is more capable to remediate Zn than *Thlaspi caerulescens* which is commonly known as Zn hyperaccumulator. The reason behind this fact is that the production of biomass in *B. juncea* is 10-times greater than in *T. caerulescens* (Baker et al., 1994).

Phytodegradation or *rhizodegradation* is the degradation activity of contaminants by specific enzyme activities. When phytodegradation specifically occurs in the rhizosphere, the process is called rhizodegradation and is a symbiotic relationship that has evolved between plants and microbes and is due to the presence of proteins and enzymes produced by them (Vidali, 2001). Phytodegradation is the main technique when the pollution in question predominantly consists in organic compounds (Fiorentino et al., 2018).

Phytovolatilization is the technique in which plants absorb contaminants from the soil and, subsequently, volatilize them into the atmosphere (Lee et al., 2020) Volatile metal(loid)s like As, Hg and Se can be removed by this technique. But it is a controversial phytoremediation strategy because contaminants are redeposited from atmosphere to soil (Iqbal and Nasrullah, 2019). It has been revealed that *Brassica juncea* can be used to volatilize Se because the plant is capable to assimilate Se from soil, convert Se to volatile methyl sclenate and release it into the atmosphere (Gupta and Gupta, 2016). In *Pteris vittata*, As is effectively evaporated in the form of arsenite/arsenate (Singh et al., 2017). Transgenic *Arabdopsis thaliana* and *Nicotiana tabacum* plants, engineered with bacterial organomecurial lyase (merB) and mercuric reductase (merA) genes, were also able to absorb elemental mercury (Hg II) as well as methyl mercury from the soil and convert it into a volatile form (Hg0) (He et al., 2001).

1.5 Factors affecting phytoremediation

The use of plants to remediate a polluted soil seems an attractive approach to tackle metal(loid) contamination problems. However, there are several factors which can affect the effectiveness of metal(loid) phytoremediation (Fig. 6), and only by having knowledge about these factors, the phytoremediation performance can be greatly improved (Tangahu et al., 2011). Environmental factors, soil physicochemical properties, metal(loid) type and concentrations, and plant species are all aspects to take in consideration for a phytoremediation approach (Sarwar et al., 2017).

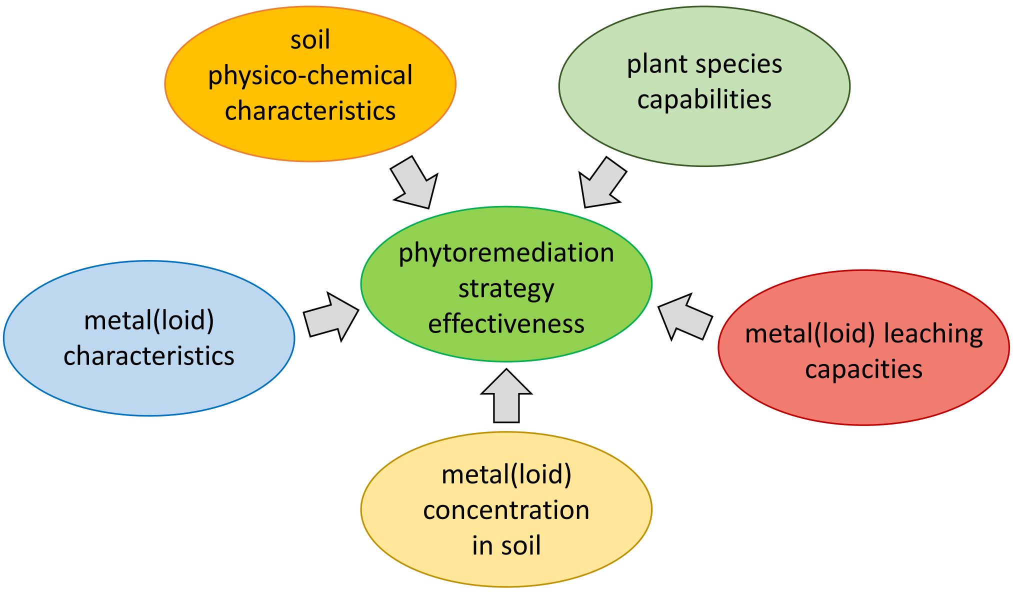

Fig. 6 ***Aspects which influence phytoremediation effectiveness***

Among environmental factors which influence phytoremediation, temperature is very important because it controls transpiration, water chemistry, growth and metabolism of plants, and therefore affects also metal(loid) stabilization, uptake and elimination (Bhargava et al., 2012). The removal rate of metal(loid)s by plants seems to increase linearly by increasing temperature (Yu et al., 2010).

Various soil characteristics such as moisture and nutrient content, pH and cation exchange capacity, can influence metal(loid) properties and metal(loid) bioavailability to plants, and in turn affect plant growth (Rosenfeld et al., 2018). For example, it is well known that the moisture content of soil influences some metabolic pathways (*e.g.*, photosynthesis and autotrophic respiration) of accumulator plants (Ghori et al., 2016). Plants produce greater biomass at higher soil moisture levels, that further enhances the amount of metal(loid) compounds able to be extracted from soil (Marchiol et al., 2004). Moreover, metal(loid) solubility and bioavailability are strongly influenced by soil pH (Sheoran et al., 2016). Metal(loid) cations like Cd, Cu, Cr, Fe, Hg, Mn, Pb, and Zn are reported to be more soluble and bioavailable in soil solutions at low pH (Ma et al., 2016), in contrast, metal(loid) anions like As and Se exhibit high mobility at high pH (Kim et al., 2015). In addition, the mobilization and bioavailability of metal(loid)s in soil are affected by cation exchange capacity and organic matter content (Sheoran et al., 2016). For instance, organic matter is characterized by negatively charged sites which on one hand may stabilize metal(loid) cations through complexation and adsorption reactions, and on the other hand may mobilize metal(loid) anions by desorption reactions (Bakshi and Banik, 2018).

The success of a phytoremediation technique depends also upon the identification of suitable plant species for a particular metal(loid) or a combination of metal(loid)s (Lorestani et al., 2012). For example, Tariq and Ashraf (2016) studied the phytoremediation potential of *Brassica campestris*, *Helianthus annuus*, *Pisum sativum* and *Zea mays* in a soil multi-contaminated by Cd, Cr, Co, Cu, Ni, and Pb. Results showed that these hyperaccumulator plants possessed different accumulation potential for different metal(loid)s. Indeed, *B. campestris* hyperaccumulated Cr, *H. annuus* exhibited hyperaccumulating properties for Cd, *P. sativum* has affinity for Pb, and *Z. mays* was the best accumulator of Co and Cr.

Even today, because of these many aspects that affect the efficiency of contaminated soil remediation, phytoremediation techniques lack large-scale applications (Sarwar et al., 2017). Indeed, traditional phytoremediation approaches have several limitations such as the long time required to remediate metal(loid) polluted soils (Tangahu et al., 2011). In order to overcome these limitations and ensure large-scale application of phytoremediation, researchers are improving traditional approaches (Fig. 7) giving rise to the new concept of “assisted phytoremediation” (Sarwar et al., 2017).

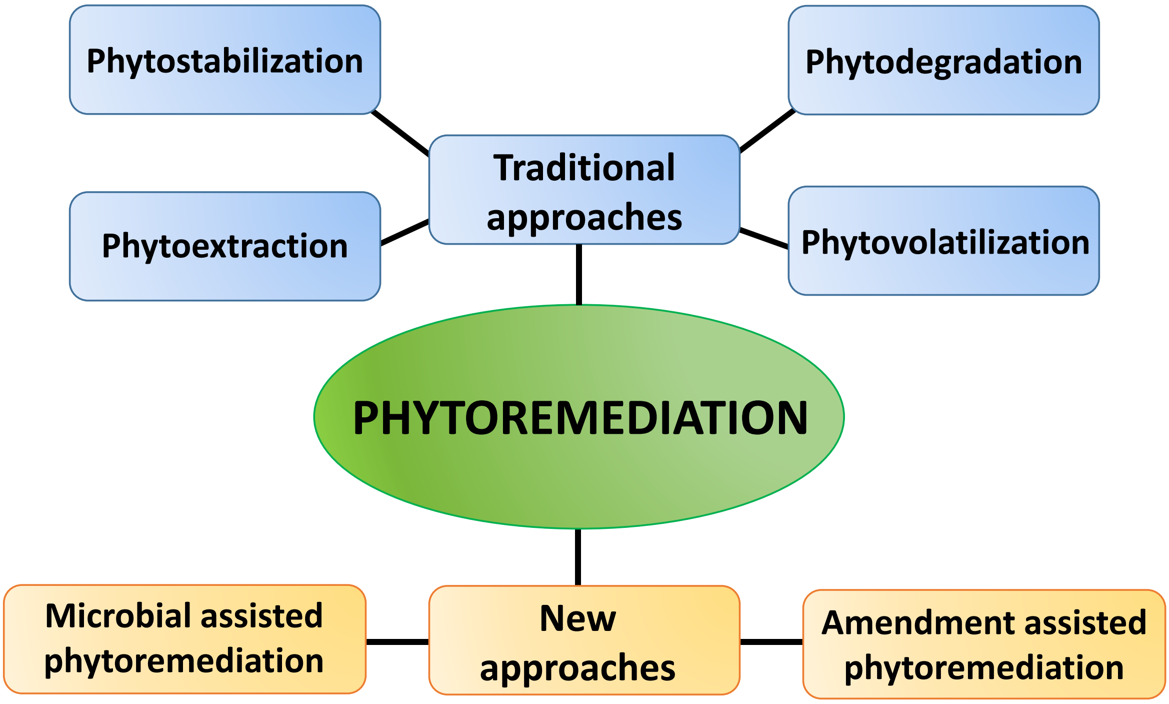

Fig. 7 ***Traditional and new approaches for soil remediation***

1.6 Assisted phytoremediation

To speed up the phytoremediation process of metal(loid) contaminated soils, the combined use with soil amendments and/or microorganisms has resulted in a faster and more proficient clean-up process (Fiorentino et al., 2018). Indeed, phytoremediation can be effective only where environmental conditions permit plant growth and activity, and its application often involves the manipulation of soil parameters to allow plant growth to proceed at a faster rate (Wu et al., 2016). Phytoremediation efficiency can be enhanced through two main techniques: (i) amelioration of soil characteristics and plan growth through amendment use, and (ii) enforcement of the phytoremediation activity with soil bacteria and fungi (Fiorentino et al., 2018). In last instance, where soil is contaminated with multiple types of metal(loid)s, application of plants, amendments and metal(loid) tolerant and plant growth promoting microorganisms is found to be more useful to phytoremediate lands (Touceda-González et al., 2015).

1.6.1 Amendment assisted phytoremediation

In the assisted phytoremediation, one of the strategies used to make soil favourable for plant growth involves the use of amendments (Fiorentino et al., 2018).

Soil amendments are materials added to a soil to improve its physicochemical properties, and they are categorized in inorganic and organic amendments (Gomes et al., 2016). The combined use of inorganic and organic amendments could be a promising choice to assist phytoremediation of contaminated soils by decreasing metal(loid) leaching and availability while improving soil fertility and plant growth (Oustriere et al., 2017).

Some inorganic amendments (*e.g.*, iron sulphate, red mud, vermiculite) are known to decrease the metal(loid) availability by enhancing adsorption, precipitation, and complexation reactions onto soils (Zhou et al., 2017; Zhao et al., 2020). Similarly, to inorganic amendments, the organic ones (*e.g.*, activated carbon, biochar, compost, manure) have a large capacity to interact with metal(loid) ions and mobilize, immobilize or adsorb them through ion exchange, surface complexation, and precipitation (Alam et al., 2020). Moreover, organic amendments are effective to improve soil physicochemical characteristics by numerous ways (Hagemann et al., 2018). Such amendments can increase the pH of acidic soils and ameliorate soil texture (Sarfraz et al., 2019), improve soil aeration, and optimize water holding capacity and nutrient retention (Lomaglio et al., 2017). Indeed, these soil amendments can enhance the availability of essential nutrients such as nitrogen, carbon, and phosphorus (Sun et al., 2018) demonstrating positive effects on soil fertility, and consequently on plant and soil microorganism growth (Wu et al., 2016).

For the purpose of this doctoral research, below the focus is reported on two organic amendments (biochar and activated carbon) and an inorganic soil amendment (red mud).

1.6.1.1 Biochar

To restore metal(loid) contaminated sites, biochar is considered as a potential soil amendment. It is a carbonaceous product obtained through the pyrolysis of biomass (*e.g.*, wood, grass, dairy manure, broiler litter, and crop residues) in the partial or total absence of oxygen (Hagemann et al., 2018). The properties of biochar depend upon the type of feedstocks and pyrolysis conditions (Sarfraz et al., 2019). However, despite the type of biochar used in an assisted phytoremediation process, biochar has the abilities to immobilize metal(loid)s in contaminated soils while improving soil quality, and it can significantly increase plant growth and reduce plant uptake of metal(loid)s (Sun et al., 2018).

Biochar is able to effectively bind metal(loid)s through its abundant surface functional groups (*e.g.*, hydroxyl and carboxylic groups), high porosity, and large specific surface area (Lomaglio et al., 2017). Moreover, biochar can absorb or combine soil metal(loid)s through several mechanisms such as complexation, reduction, cation exchange, electrostatic attraction, and precipitation functions, or convert metal(loid)s from inorganic to organic states, which changes contaminant mobility and bioavailability (Wang et al., 2018). The biochar large specific surface area available for sorption helps to form metal(loid)-biochar complexes either by exchange of metal(loid) cations with other cations (*e.g.*, Na^+, K^+, Ca^{2+}, Mg^{2+}) at biochar functional groups or by physical adsorption (Sarwar et al., 2017). Besides, biochar alkalinity may decrease the bioavailability of metal(loid)s and increase their precipitation in soil (Cheng et al., 2020).

The improvement in soil properties following biochar addition regard amelioration of soil texture and structure, increasing of soil organic matter and water holding capacity, raising of nutrient availability, and consequently stimulating soil microbial activity and crop yield (Sarfraz et al., 2019). By immobilizing metal(loid)s in soil and increasing plant biomass, biochar can decrease metal(loid) uptake by plants and dilute metal(loid) content in plant tissues reducing, in turn, their phytotoxicity (Cheng et al., 2020).

Meier et al. (2017) indicated that 5% chicken-manure-derived biochar reduced the uptake of Cu from 67 $mg \cdot kg^{-1}$ to 37 $mg \cdot kg^{-1}$ in the aboveground part of *Oenothera picensis* plants in copper-mine-polluted soil, and increase the biomass of shoots and roots by 4 times and 3 times, respectively.

Similarly, Park et al. (2011) reported that 1% chicken-manure-derived biochar increased *Brassica juncea* dry biomass by 353% and 572% for shoots and roots, respectively, in a Cd, Cu and Pb polluted soil. This was attributed to reduced toxicity of metal(loid)s and increased availability of nutrients such as P and K. The authors also showed that biochar significantly reduced Cd, Cu and Pb accumulation by plants, and this reduction increased with increasing biochar application rate. Li et al. (2018) applied 3% of soybean-straw-derived biochar to a multi-contaminated soil by As and Cd, which reduced the bioaccumulation of As in rice plants by 88%.

1.6.1.2 Activated carbon

Activated carbon is defined a pyrogenic carbonaceous material since it is produced by thermochemical process and contains organic carbon (Hagemann e al., 2018). It is obtained from carbonaceous materials through two phases of manufacturing: pyrolysis and activation (Abdulrazak et al., 2017). As above mentionated for biochar, some properties of activated carbon depend upon pyrolysis conditions based on temperature, duration and presence or absence of oxidants (Hagemann et al., 2018). Moreover, the type of activation also influences activated carbon characteristics. The activation process can be performed either with chemical agents (*e.g.*, $ZnCl_2$, KOH, and H_3PO_4) or with physical activation by steam (Hagemann et al., 2018). Activated carbon can be produced by organic materials of plant origin (*e.g.*, coal, coconut shells and wood, sugarcane bagasse, soybean hulls and nutshell) and, on a limited scale, by animal manures. The use of activated carbon is widely common to remove metal(loid)s from waste waters, more recent are instead the studies on activated carbon use in phytoremediation of contaminated soils (Gupta et al., 2015). Activated carbon has excellent capacity to adsorb metal(loid)s due to its porous structure and large specific surface area (Lima and Marshall, 2005). In addition, also organic matter obtained from the dissolution process of activated could also retain metal(loid)s by forming stable complexes with them (Clemente et al., 2006). Activated carbon capacity to stabilize metal(loid)s into soil is also influenced by soil pH that affects mobility and phytoavailability of metal(loid)s (Abdulrazak et al., 2017). Generally, addition of activated carbon to contaminated soil increases soil pH, and it is known that metal(loid) retention raises with increasing pH (Bolan and Duraisamy, 2003). In addition to metal(loid) immobilization, activated carbon is able to ameliorate soil fertility increasing the availability of some plant essential nutrients with the consequential increasing of plant growth (Chamon et al., 2005). Sabir et al. (2013) observed that 4% of activated carbon significantly increased *Zea mays* dry weight in a multi-contaminated soil by Cu, Fe, Mn, Ni, Pb and Zn. Activated carbon was also effective in immobilization of Cu, Mn, Ni, and Zn in the soil, and in decreasing plant shoot Mn concentration by 27% and Ni concentration by 48% compared with the control.

1.6.1.3 Red mud

Red mud, also called bauxite residue, is a by-product of alumina production from bauxite by the Bayer process (Akinci and Artir, 2008). Bauxite mineralogy and the different Bayer process phases affect chemical and physical properties of red mud (Paramguru et al., 2005). However, on average, the composition of red mud is characterized by large amounts of Fe_2O_3 (41%) and Al_2O_3 (17%), smaller amounts of SiO_2 (10%), TiO_2(9%), CaO (9%) and Na_2O (5%), and trace of metal(loid)s (*e.g.* Cr, Cu, Pb and Zn) and radionuclides (Liu et al., 2007). Red mud is an alkaline product with a pH ranging between 9 and 13 and has a high electrical conductivity ranging from 0.7 to 61 $mS \cdot cm^{-1}$ (Udeigwe et al., 2009). Although red mud has always been treated as waste and stored in large lagoons or in land-based disposal pits, it has recently been considered for its potential as a soil amendment for the remediation of contaminated soils characterised by high metal(loid) concentrations and low pH (Lombi et al., 2002). Red mud contains small amounts of potential toxic elements that could be a problem when it is used as an amendment, however several studies have shown that the mobility of these toxic elements in red mud is very low (Rubinos and Barral, 2013). Thus, red mud can be effectively used for the restoration of metal(loid) contaminated soils. Indeed, it has been observed that when red mud is added to polluted soils, mainly resulting from mining activities, it is able to neutralize soil low pH and reduce metal(loid) mobility and availability through different physicochemical mechanisms (Santona et al., 2006). Several factors control the effectiveness of red mud for metal(loid) immobilisation in contaminated soils. Soil pH plays a significant role in metal(loid) mobility. In the majority of cases, metal(loid) polluted soils have a low pH, thus the addition of red mud can increase soil pH and consequently reduce metal(loid) mobility (Huang and Hao, 2012). Moreover, mobility reduction of metal(loid)s such as Cd, Cu, Pb and Zn is related to increased metal(loid) adsorption due to the increasing negative surface charge of soil and precipitation of metal(loid) hydroxides and carbonates at high pH (Appel and Ma, 2002). Mobility of As responds differently to pH, with experiments showing that arsenate adsorption by red mud decreased as pH increased (Castaldi et al., 2010). Nevertheless, pH change is not the only factor involved in soil metal(loid) stabilisation after addition of red mud. Red mud composition also plays an important role in the immobilisation of metal(loid)s due its high Fe and Al content (Feigl et al., 2012). Typically, 60% by weight of red mud comprises Fe and Al oxides and oxyhydroxides as hematite, boehmite, gibbsite, and cancrinite (Ahn et al., 2015). These molecules have a high sorption capacity for metal(loid)s which can be immobilized on the reactive surface of red mud forming some molecular complexes with the Fe and Al oxide and oxyhydroxide phases (Santona et al., 2006). Fe and Al oxide and oxyhydroxide phases also play an important role in As immobilization. Indeed, response of As in contaminated soil amended with red mud may appear as either immobilisation through the Fe and Al oxide content of red mud, or increased As mobility due to a high pH (Lee et al., 2011).

By immobilizing metal(loid)s into soil and reducing their availability, remediation of contaminated soils with red mud has been also shown in a number of studies to decrease metal(loid) concentrations in plants (Hua et al., 2017). Lee et al. (2011) observed that metal(loid) concentration in *Lactuca sativa* grown in soil amended with 2% red mud decreased by 33% (As), 84% (Cd), 35% (Pb) and 81% (Zn), compared to lettuce grown in non-amended soil. Castaldi e al. (2009) showed that *Pisum sativum* and *Triticum vulgare* plants grown on a contaminated soil amended with red mud accumulated significantly lower amounts of Cd, Pb and Zn with respect to plants grown in non-amended soil. Moreover, the authors observed that red mud amendment increased the proportion of metal(loid) accumulation in *P. sativum* and *T. vulgare* plant roots compared with the one in plant shoots.

In many studies, contaminated soil remediation with red mud also increased plant biomass (Hua et al., 2017). For example, in a Cu contaminated soil, *Zea mays* biomass was increased by 130% when grown in red mud amended soil compared with plants grown in non-amended soil (Friesl et al., 2004). Furthermore, in both pot and field experiments with 5% red mud application rate in a contaminated soil, Pb concentration in *Festuca rubra* decreased while the biomass yield of plants increased by more than 100% compared to the control (Gray et al., 2006).

1.6.2 Microbial assisted phytoremediation

In assisted phytoremediation, in addition to the use of amendments, another strategy to improve soil quality, and enhance plant growth and plant capacity to tolerate, remove or stabilize metal(loid)s, involves the use of microorganisms (Yang et al., 2020). Soil microorganisms – in particular rhizosphere microorganisms – show abilities to protect plants from metal(loid) stress as well as help them in removal of these contaminants (Sarwar et al., 2017).

Plant growth promoting rhizobacteria (PGPR) are an important microbial community that can be divided as symbiotic bacteria and free-living rhizobacteria (Mishra et al., 2017).

Symbiotic as well as free-living PGPR reside in the soil environment around plant roots where they are able to protect the host plants against pathogens and contaminant, and can increase plant growth by various direct or indirect mechanisms (Nadeem et al., 2014). Direct mechanisms include siderophore production, phosphate solubilisation, 1-aminocyclopropane-1-carboxylate deaminase synthesis, and indole-3-acetic acid production which allow plants to withstand abiotic stress conditions (Ahemad, 2014). Indirect mechanisms of plant growth promotion involve the PGPR acting as biocontrol agents to detoxify metal(loid)s (Glick, 2012).

Siderophores are small, high-affinity iron-chelating compounds secreted by microorganisms and plants, especially under Fe-limiting conditions. Iron is one of the essential nutrients required for plant metabolism and when Fe is limited in soil, microbial siderophores solubilize and remove Fe from the soil and supply plants with this nutrient, thereby enhancing their growth (Mishra et al., 2017).

Phosphorous is required by plants as a macronutrient but it reacts naturally with Al, Ca, and Fe, becoming unavailable to plants. Some PGPR are capable to convert insoluble phosphorous to a soluble form supplying phosphorous to plants under stress conditions. Phosfate-solubilizing microorganisms include *Azotobacter*, *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Rhizobium* (Chandra and Singh, 2016).

Many PGPR directly stimulate plant growth by synthesizing the 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme cleaves and sequesters 1-aminocyclopropane-1-carboxylic acid (ACC) that plays an important role in the biosynthesis of the plant hormone ethylene. PGPR with ACC deaminase activity breakdown ACC preventing toxic level of ethylene accumulation in order to avoid plant death (Ojuederie and Babalola, 2017).

Several microorganisms isolated from the rhizosphere of different crops also have the capacity to produce and release auxins as secondary metabolites (Ahemad, 2014). Indole-3-acetic acid (IAA) is synthesized by PGPR using tryptophan obtained from plant roots (Glick, 2012). According to Spaepen and Vanderleyden (2011), plant growth and development is regulated by exogenous levels of IAA since a low amount enhances root elongation and a high amount decreases primary root length, stimulate formation of lateral roots and increases root hair formation. The production of IAA is very important in plant-microbe relations because the IAA synthesized by rhizobacteria enhances root elongation and surface area, making soil nutrients more available and thereby enhancing root exudates which gives the rhizosphere bacteria more nutrients for their activity (Glick, 2012).

Mycorrhizal fungi are another important soil microbial community that exhibits a symbiotic relationship with their host plants. This relationship promotes plant growth, it helps to remediate metal(loid) contaminated soils, and thus enhance phytoremediation process (Sarwar et al., 2017). Among mycorrhizal fungi, arbuscular mycorrhizal fungi (AMF) are widely distributed in rhizosphere (Yang et al., 2020). AMF establish direct physical link between soil and plant roots which increase root surface area facilitating nutrient absorption by plants (Tiwari and Lata, 2018). AMF are also involved in alleviating metal(loid) toxicity to the host plant by several mechanisms including metal(loid) bound to AMF cell wall and deposit in the vacuoles, and metal(loid) sequestration by the help of siderophores into soil (Sarwar et al., 2017). Moreover, AMF can use two different strategies to enhance phytoremediation. The first strategy consists in increasing root absorptive area and enhancing plant capacity to take up metal(loid)s; while the second one regards the AMF hypha production of organic acids, glycoproteins and cyclosporine to form metal complexes that reduce the amount of metal(loid)s translocated from plant roots to shoots (Yang e al., 2020).

Several studied showed how the inoculation of plants with metal(loid) resistant microorganisms, and root-associated beneficial bacteria and fungi improve plant survival and thereby enhance phytoremediation in metal(loid) polluted soils.

Ma et al. (2009) demonstrated that *Brassica juncea* inoculated with a Cu-resistant strain of *Achromobacter xylosoxidans* exhibited an increase in plant dry weight by 97%, 53% and 6% as Cu concentration increase in soil (50 $mg \cdot kg^{-1}$ soil, 100 $mg \cdot kg^{-1}$, and 150 $mg \cdot kg^{-1}$, respectively). The bacterial strain also stimulated Cu accumulation by plants, and maximum accumulation of Cu in *B.*

juncea roots and shoots was 180 and 8 mg·kg^{-1}, respectively, at the highest Cu concentration of 150 mg·kg^{-1}, whereas non-inoculated plants accumulated 42 mg·kg^{-1} and 4 mg·kg^{-1}, respectively.

An As hypertolerant bacterium *Staphylococcus arlettae* strain significantly increased root length (93%), shoot length (58%), dry weight (100%), protein (44%), chlorophyll (95%), and carotenoid content (50%) in *B. juncea* in an As contaminated soil (15 mg·kg^{-1} of As). In addition, As concentration in shoots and roots of inoculated plants increased from 4% to 34% and from 87% to 99%, respectively, compared with the non-inoculated plants (Srivastava et al., 2013).

Similar observations were made by Zhang et al. (2011) that observed an increase of dry weight and Pb content in *Brassica napus* inoculated with a Pb resistant endophytic bacteria (*Acinetobacter* sp. and *Bacillus* sp.) compared with the control. *B. napus* dry weight of aboveground biomass and roots increased, respectively, by 71% and 123% compared with the non-inoculated plants, in a soil containing 100 mg·kg^{-1} of Pb. Moreover, the Pb content in plants increased from 58% to 62% in inoculated plants compared with the control.

In Cd, Ni and Zn multi-contaminated soils, AMF inoculation increased the growth of both *Avena sativa* roots and shoots by up to 70% and 55%, respectively. Thus, mycorrhization also led to higher concentrations of Cd, Ni and Zn in plant roots (Ricken, 1996).

Arriagada et al. (2004) reported that the dual inoculation with a fungus (*Trichoderma koningii*) and an arbuscular mycorrhizal fungus (*Glomus deserticola*) increased the *Eucalyptus globulus* dry weight, compared with the control, in presence of 50 mg·L^{-1} of Cd. Moreover, the AMF inoculation led to a raised accumulation of Cd in the eucalyptus.

1.7 Plant selection for phytoremediation strategies

Environmental features, soil properties, contamination type, and plant characteristics are all factors that must be taken into consideration in the plant species selection for phytoremediation purpose, whether it is a traditional or more recent assisted phytoremediation approach (Ghosh and Singh, 2005).

Indeed, preferably, plants chosen for phytoremediation of metal(loid)s from any contaminated site should have the following characteristics: (i) tolerate high concentrations of metal(loid)s, (ii) accumulate high amounts of metal(loid)s in their tissues, (iii) have a rapid growth rate, (iv) have a high biomass production, and (v) possess a large and deep root system (Mahar et al., 2016).

A large number of both hyperaccumulator and non-hyperaccumulator plants, including grasses, vegetable crops and trees, have been reported as suitable plants to use in phytoremediation (Leguizamo et al., 2017).

As described previously in this thesis, hyperaccumulator plants are able to accumulate high amounts of metal(loid)s in their aboveground parts and, in turn, have been and still are widely used for the metal(loid) phytoextraction from polluted soils (Ghori et al., 2016). Over 400 herbaceous and small woody species have been identified as hyperaccumulator plants and they belong to several families such as *Asteraceae, Brassicaceae, Cyperaceae, Euphorbiaceae, Fabaceae, Flacourtiaceae, Lamiaceae, Poaceae,* and *Violaceae* (Singh et al., 2017). The *Brassicaceae* family is relatively rich in hyperaccumulators species, indeed, about of 25% of hyperaccumulator plants belong to this family, and specifically to *Alyssum* and *Thlaspi* genera (Rascio and Navaiizzo, 2001). Numerous high biomass crop plants, such as oat (*Avena sativa*), Indian mustard (*Brassica juncea*), cabbage (*Brassica rapa*), sunflower (*Helianthus annuus*), pea (*Pisum sativa*), tobacco (*Nicotiana tabacum*) and maize (*Zea mays*), have been studied by researchers for their ability to accumulate metal(loid)s from polluted soils (Cui et al., 2004; Szabó and Fodor, 2006).

Even if metal(loid) hyperaccumulator plants are useful in soil cleanup, as they can uptake significant amounts of metal(loid)s, often their slow growth rate and low annual biomass production tend to limit the phytoextraction capability (Ghori et al., 2016).

Thus, non-hyperaccumulator and excluder plants are a possible alternative in phytoremediation strategies due to their higher biomass production and fast growth rate (Ghosh and Singh, 2005). Indeed, over the last few years, trees have received increasing attention for their use as suitable vegetable cover for contaminated areas (Pulford and Watson, 2003).

Trees have many features that make them a good option in phytoremediation technologies, such as (i) the capacity to establish on a site with low soil fertility, (ii) a fast growth rate, and (iii) a massive root system that can penetrate soil for several meters (Ghori et al., 2019). Many studies have been conducted on different tree species able to grow on metal(loid) polluted soils. In detail, researchers have been focused the attention on genera as *Acer* (sycamore), *Alnus* (alder), *Betula* (birch), *Populus* (poplar), and *Salix* (willow). Among them, for example, both *Populus* spp. and *Salix* spp. are fast growing plants that have high biomass production and extensive root system with elevated rates of water uptake and transpiration. All these features can result in a very efficient metal(loid) transport from roots to shoots (Pilipovic et al., 2019). Moreover, the large and deep root apparatus of *Populus* and *Salix* plants is able to prevent erosion of contaminated sites by wind (Janssen et al., 2015).

Despite not having the ability to hyperaccumulate metal(loid)s, non-hyperaccumulator plant species can be used in phytoremediation for their capacity to extract metal(loid)s from soil and stabilize these contaminants in rhizosphere area or into their roots (Ghori et al., 2016).

Metal(loid) extraction from a site can be higher in non-hyperaccumulator than hyperaccumulator plants because the biomass production of the first overcomes by several order of magnitude the phytoremediation capacities of typical herbaceous hyperaccumulator species (Pulford and Watson, 2003).

Usually, native plants that are involved in the uptake of metal(loid)s, both with or without hyperaccumulator ability, can provide the most reliable method for the extraction of metal(loid)s from a polluted land (Chandra and Kumar, 2017). It could be efficient to use the native plants of contaminated soils for phytoremediation, because these plants are naturally adapted in terms of survival, growth and reproduction under environmental stresses with respect to plants introduced from another environment (Fernandez et al., 2017). Chandra and Kumar (2017) collected 15 native plants growing on stabilized distillery sludge and found that all of them were able to accumulate Fe, Mn and Zn in different plant organs. However, particular non-native plants may work best remediation of specific contaminants compared to native species and can be safely used under circumstances where the possibility of invasive behavior has been eliminated (USEPA 2000).

1.8 *Arabidopsis thaliana* as model organism for phytoremediation studies

The great expansion, over the past few years, of public and private interest on phytoremediation and assisted phytoremediation potential in reducing the impact of human activity on the environment, has strongly fostered both applied and basic research discoveries. Indeed, beside applied studies, there has been growing interest also in basic research to shed more light on mechanisms involved in metal(loid) tolerance in plants (Jabeen et al., 2009). Additionally, further basic research is needed to better understand the complex interactions among soil, metal(loid)s, plants and microorganisms in the rhizosphere area in order to fully exploit the metabolic diversity of plants and thus successfully implement phytoremediation technology (Dubey et al., 2018).

In order to comprehend the complex strategies used by plants at the cellular and molecular level to overcome the stress condition due to the presence of high level of metal(loid)s, numerous studies have been conducted by using model plants (Cobbett and Meagher, 2002).

A model organism is a species that has been extensively studied in order to understand particular biological phenomena, on the assumption that findings made on the model organism can provide insights into other organisms (Koornneef and Meinke, 2010). This is possible because most biological characteristics, such as metabolic and developmental processes, as well as the genes that regulate them, are conserved throughout evolution (Pace et al., 2012).

The most commonly used model plants belong to the *Populus* genus, in the case of tree species, and *Arabidopsis* genus, in the case of herbaceous species (Chang et al., 2016).

The development of *Populus* as a model organism for the biology of trees and woody perennial plants has been largely due to the rapid development of genomic and molecular biology resources for this genus, culminating in the sequencing of the *Populus trichocarpa* (black cottonwood) genome (Tuskan et al., 2006). *P. trichocarpa* has several characteristics that make it a useful model plant: (i) a small genome size (about 400 million base pairs), (ii) adaptation to different environments, (iii) easy propagation, (iv) abundant genetic variation in natural populations, and (v) facile transformation and regeneration to create transgenic poplars (Jansson and Douglas, 2007). However, due to its large size and long generation interval, poplar is not easy to use in laboratory experiments requiring short time spans (Bradshaw et al., 2000).

Thus, *Arabidopsis,* in particular *Arabidopsis thaliana,* is often used as a model plant in many basic research experiments to overcome the limitations posed by the *Populus* genus (Chang et al., 2016). *A. thaliana* emerged as model organism for plant biology, thirty-five years ago, and since then an intensive effort from the plant research community has allowed the development of extensive genomic resources, tools, and techniques that have, in turn, provided a wide understanding of many important biological processes in plants (Cobbett and Meagher, 2002). Indeed, *A. thaliana* became the model plant for excellence (Fig. 8), thanks to its small genome size, short life cycle, high fecundity, small physical size, considerable number of ecotypes and mutants (Jansson and Douglas, 2007), and the wide availability of databases with genomic, transcriptomic and proteomics information (Dubey et al., 2018). The small size genome with about 125 million base pairs of *A. thaliana* has been completely sequenced (Arabidopsis Genome Initiative, 2000), and it serves as an ideal model for the larger and more difficult genomes of native grass, shrub, and tree species usually used in phytoremediation (Cobbett and Meagher, 2002). Due to their small size, *A. thaliana* plants can be grown in petri plates or maintained in pots located either in a greenhouse or under fluorescent lights in the laboratory (Meinke et al., 1998). The *A. thaliana* entire life cycle, including seed germination, formation of a rosette plant, development of the main stem, flowering, and maturation of the first seeds, is completed in 6 weeks (Meyerowitz and Somerville, 1994). Moreover, many different ecotypes have been collected from natural populations and are available for experimental analysis (*e.g.*, the Columbia and Landsberg ecotypes are the accepted standards for genetic and molecular studies) (Meinke et al., 1998). Additionally, *A. thaliana* provided useful information about the physiological and molecular mechanisms regulating plant metal(loid) tolerance, and it revealed to be also an excellent model organism to test foreign gene expression (Xu et al., 2001).

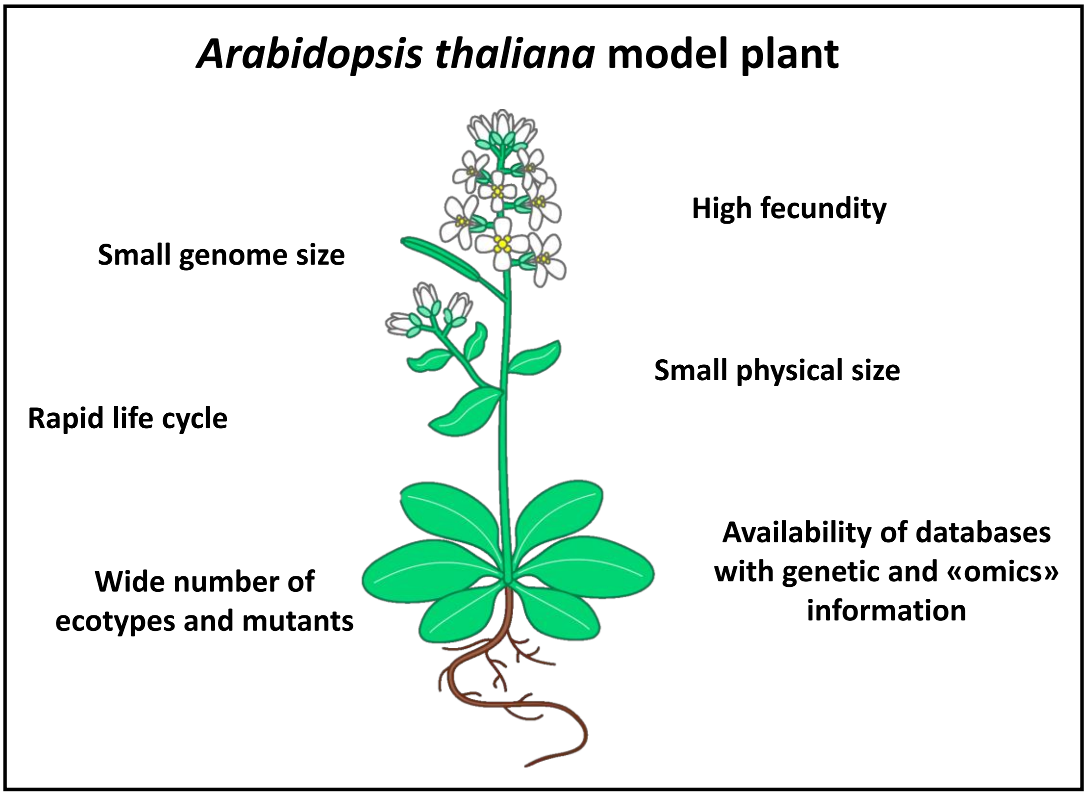

Fig. 8 ***Arabidopsis thaliana model plant characteristics***

Several studies on response mechanisms to metal(loid)s have shown that hundreds of *A. thaliana* genes encode transport, detoxification, and sequestration proteins with potential roles in phytoremediation (Abercrombie et al., 2008). Furthermore, results of proteomics studies suggested that differentially expressed proteins involved in providing tolerance against metal(loid) toxicity are related to the protein biosynthesis, cell signalling, stress, detoxification, defence, and development mechanisms (Khan et al., 2014). Proteomics analyses have also confirmed that metal(loid) stress in plants induces reactive oxygen species, activating signalling molecules such as jasmonic acid-inducible protein, and auxin-inducible protein, and triggering the detoxification process which mostly involves glutathione and phytochelatin biosynthesis (Hossain et al., 2012). For example, Smith et al. (2004) conducted a comparative proteomic analysis of the *Arabidopsis* response to Cu and herbicide stresses. In comparison with control and herbicide-treated plants, Cu-treated samples showed elevated levels of four glutathione S-transferases (GSTs) suggesting that these GSTs have some specific functions in detoxifying Cu in plant cells. In a similar way, Sarry et al. (2006) observed that the main response of *Arabidopsis* to Cd toxicity was activation of carbon, nitrogen, and sulphur metabolisms to trigger the synthesis of chelating molecules such as phytochelatin or its precursor glutathione. Guo et al. (2008) studied the role of chelating proteins in resistance to Cd and Cu using *Arabidopsis* plants mutant for some genes encoding metallothioneins and phytochelatins. The authors observed that metallothionein deficiency combined with phytochelatin deficiency made *Arabidopsis* sensitive to both metals. These results provided evidence that these proteins function cooperatively to protect plants from Cd and Cu toxicity. Transcriptomic analysis of *A. thaliana* exposed to Hg was studied by Heidenreich et al. (2001). The study reported that Hg-induced genes encode proteins involved in P450-mediated biosynthesis of secondary metabolites, cell wall metabolism, and chlorophyll synthesis. Moreover, several metabolomics studies have been carried out to analyse content of many metabolites under metal(loid) exposure in *Arabidopsis* (Wang et al., 2015).

All these functional genomic and "omics" studies, in combination with the study of model plants (*e.g.*, *A. thaliana*), provided important information for the development and the identification of varieties/ecotypes with enhanced tolerance to metal(loid)s, which may potentially be used in environmental decontamination programmes (Mosa et al., 2017).

Indeed, thanks to these approaches, several genes involved in the tolerance to different metal(loid)s have been identified and they might be used for plant improvement by assisted selection (Zhang et al., 2017). More knowledge at the molecular level may also open up new possibilities for phytoremediation by improving plants through genetic engineering, for example by modifying characteristics such as metal(loid) uptake, transport and storage, and plant tolerance to metal(loid)s (Gajac et al., 2018). For example, transgenic *A. thaliana*, overexpressing the zinc transporter NcZNT1, showed enhanced Zn tolerance as well as increased accumulation of Zn and Cd in comparison with wild-type plants (Lin et al., 2016). Calcium vacuolar transporter, cation/proton exchanger 2 (CAX-2) from *Arabidopsis*, causes enhanced accumulation of Ca, Cd, and Mn when overexpressed in tobacco (Pittman and Hirschi, 2016).

Moreover, the use of the *A. thaliana* model plant and the recent "omics" approaches provided first insights for understanding the effects of soil amendments on plant gene expression (Viger et al., 2014), and the interactions between plants and soil microbial communities (Cole et al., 2017; Pascale et al., 2019).

Thus the combinations of "omics" tools and bioinformatics approaches on the *A. thaliana* model organism, may allow a deep understanding of integrated activity patterns between plants, amendments, and microbes, in different environmental soil conditions. The results of these studies may be used to assess if and how plant-microorganism interaction can be modified to maximize plant growth (*e.g.*, by using different types of amendments), identify the appropriate assembly of microbial communities, and, in turn, ameliorate phytoremediation process.

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2 Objectives and papers

Soils may suffer from a large diversity of inorganic contaminants such as metal(loid)s, which can result very harmful for plants, animals and entire ecosystems. Indeed, inorganic contaminants can reduce the growth of plants by increased pollutant concentrations or endanger groundwater or surface waters.

During the past decades, numerous studies have been conducted which have led to the identification of several biological methods to mitigate the contaminant impacts and remediate the polluted soils. Under favourable conditions, appropriate use of such methods can restore soils to good shape.

More recently, among several biological methodologies, the use of phytoremediation technologies assisted by soil amendments and microorganisms has proven to be a good approach for the decontamination of metal(loid) polluted soils. Depending on the specific contaminant, soil and climate conditions, the most appropriate method using plants, organic or inorganic soil amendments, and microorganisms, individually or in combination, should be selected. The vast field of potential contaminant situations requires a continued development of new remediation strategies, including combinations of already tested and proven options as well as the development of new and innovative techniques.

Indeed, although several assisted phytoremediation methods based on plants, amendments, microorganisms, and their interactions have been developed for soil remediation mainly in the laboratories, the practical application of some of them needs to be tested under "real" conditions. Not a single method can be applied to all soils, climatic and pollution situations, but there is an optimum method for each particular case.

Metal(loid)s can be removed, stabilised, immobilised, and in some cases volatilised by specific plant species, under the support of soil amendments and microorganisms to modify the availability of the contaminants and enhance phytoremediation technique. Considering the complexity of contaminant-plant-amendment-microorganism interactions in the soil, and the multitude of environmental factors affecting a successful phytoremediation, a rapid increase of assisted phytoremediation to deal with contaminated soils under field/environmental conditions is highly desirable. Generally, there is a gap between researches in laboratories under controlled conditions and a "real field scenario" where plant species grow and survive in a polluted environment. Therefore, more research on assisted phytoremediation should be carried out under real environmental conditions in order to obtain further information about the factors influencing this useful technique to restore polluted lands.

Beside the applied studies in a real field scenario, basic research aimed at providing a deep understanding of molecular mechanisms involved in plant tolerance to metal(loid) stress, plant-soil microorganism interactions, and amendment effects, have also a pivotal role for improving assisted phytoremediation technologies and restoring polluted environments.

Based on these needs, the objective of the present doctoral thesis was twofold: (i) assess the potential of different phytoremediation techniques assisted by soil amendments and microorganisms, both alone and in combination, under either controlled or semi-natural environmental conditions for their application to different soil inorganic contaminants in mining areas; (ii) provide insights for a better understanding of mechanisms used by plants to tolerate high level of metal(loid)s in the soil, of the interaction between plant and microorganisms, and the effects of soil amendments.

To accomplish these aims, three different experimental systems were used involving a woody, an herbaceous and a model plant.

In detail, the specific objectives of the three experiments, on which the doctoral thesis was based, were:

- to verify diversities among different woody plant species and assess the best modality of the biochar organic amendment application in the assisted phytoremediation of an arsenic contaminated soil, by conducting an experiment under environmental conditions (paper I);

- to investigate differences among various soil organic and inorganic amendment combinations and their influences on the growth of an herbaceous plant in an arsenic and lead polluted soil, by performing a study under controlled conditions (paper II);

- to understand molecular mechanisms and pathways underlying plant tolerance to metal(loid) stress in the multi-contaminated soil by arsenic and lead added with biochar amendment and bacteria both alone and in combination, by carrying out a basic research on the *Arabidopsis thaliana* model organism (paper III).

Below, the three papers reporting the results obtained during the doctorate program:

I. **Assisted phytoremediation of a former mine soil using biochar and iron sulphate: effects on As soil immobilization and accumulation in three *Salicaceae* species**
Melissa Simiele, Manhattan Lebrun, Florie Miard, Dalila Trupiano, Philippe Poupart, Olivier Forestier, Gabriella S. Scippa, Sylvain Bourgerie, Domenico Morabito.
(2020) Science of the Total Environment, 710: 136203.
https://doi.org/10.1016/j.scitotenv.2019.136203

II. **Evaluation of different amendment combinations associated with *Trifolium repens* to stabilize Pb and As in a mine-contaminated soil**
Melissa Simiele, Manhattan Lebrun, Giorgia Del Cioppo, Gabriella S. Scippa, Dalila Trupiano, Sylvain Bourgerie, Domenico Morabito.
(2020) Water, Air and Soil Pollution, 231: 539.
https://doi.org/10.1007/s11270-020-04908-0

III. **In-depth study to decipher mechanisms underlying *Arabidopsis thaliana* tolerance to metal(loid) soil contamination in association with biochar and/or bacteria**
Melissa Simiele, Gabriella Sferra, Manhattan Lebrun, Giovanni Renzone, Sylvain Bourgerie, Gabriella S. Scippa, Domenico Morabito, Andrea Scaloni, Dalila Trupiano.
(2020) Environmental and Experimental Botany.
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Paper I

Assisted phytoremediation of a former mine soil using biochar and iron sulphate: effects on As soil immobilization and accumulation in three *Salicaceae* species

Melissa Simiele, Manhattan Lebrun, Florie Miard, Dalila Trupiano, Philippe Poupart, Olivier Forestier, Gabriella S. Scippa, Sylvain Bourgerie, Domenico Morabito.

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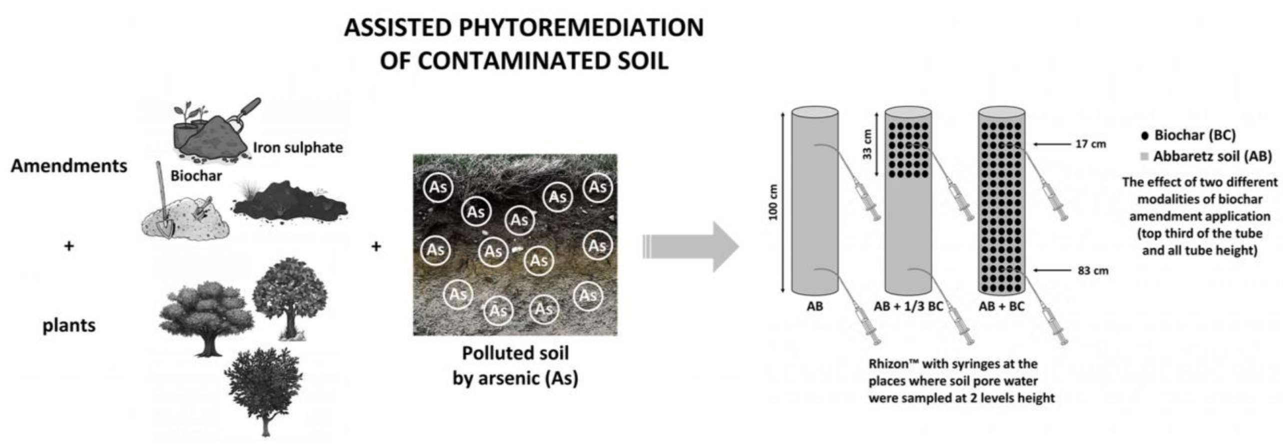
Graphical abstract



Abstract

Metal(loid) accumulation in soils, is of increasing concern because of the potential human health risks. Therefore, metal(loid) contaminated sites need rehabilitation. It is becoming increasingly popular to use phytoremediation methods for the reclamation of sites containing metal(loid)s. However, plant establishment and growth on contaminated soils can be difficult due to high metal(loid) concentrations and poor fertility conditions. Consequently, amendments, like biochar and iron sulphate, must be applied. Biochar, obtained from plant biomass or animal wastes pyrolyzed under minimal oxygen supply, showed beneficial effects on soil properties and plant growth. Iron sulphate can effectively immobilize anions, thus mitigating metal(loid) toxicity and hence promoting plant development. This study aimed to assess the effect of two different modalities of biochar amendment application (top third of the tube and all tube height) combined with iron sulphate addition on the physicochemical properties of a mining polluted soil and the growth and metal(loid) uptake of three *Salicaceae* species. A 1.5 year mesocosm experiment under field condition was conducted using a former tin mine contaminated by arsenic, amended with biochar and iron sulphate and vegetated with three *Salicaceae* species. Results showed that the combination of biochar and iron sulphate improved soil characteristics by increasing pH and electrical conductivity and reducing soil pore water metal(loid) concentrations. Between the two biochar application methods, the addition of biochar on the all tube height showed better results. But for such contaminated soil, biochar, in combination with iron sulphate, had no positive effect on plant growth, for all species tested and especially when incorporating on the top third of the tube. Finally, *S. purpurea* presented high root metal(loid) concentrations associated to the better growth compared to *P. euramericana* and *S. viminalis*, making it a better candidate for phytostabilization of the studied soil.

Paper II

Evaluation of different amendment combinations associated with *Trifolium repens* to stabilize Pb and As in a mine-contaminated soil

Melissa Simiele, Manhattan Lebrun, Giorgia Del Cioppo, Gabriella S. Scippa, Dalila Trupiano, Sylvain Bourgerie, Domenico Morabito.

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Abstract

Assisted phytoremediation using amendments is a cost-effective and environmentally friendly approach to control soil pollution. However, amendment type, combination and application rate can influence process effectiveness. In the present study, the effect of the association of red mud and carbon-based amendments on the physicochemical properties of a former mine soil as well as the growth and metal(loid) uptake of *Trifolium repens* was investigated. For this purpose, a mesocosm experiment was set up using a former mine technosol highly contaminated by As and Pb, amended with red mud combined with different carbon-based amendments, i.e., bamboo biochar, oak biochar, steam activated carbon and acidic activated carbon, and sown with *Trifolium repens*. The final goal was to determine which amendment combination allows soil metal(loid) immobilization and an efficient plant growth. Results showed that all the four different treatments improved soil characteristics by increasing pH and electrical conductivity and reducing redox potential. All the treatments were also effective in reducing soil pore water lead concentrations. Among the four treatments, the addition of red mud and acidic activated carbon in the soil showed better results regarding *Trifolium repens* growth. Finally, when grown on the soil amended with red mud and acidic activated carbon, *Trifolium repens* presented mainly a metal(loid) storage in roots, making it a right candidate for the establishment of a vegetation cover.

Paper III

In-depth study to decipher mechanisms underlying *Arabidopsis thaliana* tolerance to metal(loid) soil contamination in association with biochar and/or bacteria

Melissa Simiele, Gabriella Sferra, Manhattan Lebrun, Giovanni Renzone, Sylvain Bourgerie, Gabriella S. Scippa, Domenico Morabito, Andrea Scaloni, Dalila Trupiano.

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Abstract

Metal(loid)s are toxic to animal life, human health and plants; therefore, their removal from polluted areas is imperative in order to minimize their impact on the ecosystems. The use of plant-amendment-microorganism synergy is a promising option, but not yet fully explored, to manage lands contaminated with metal(loid)s. However, molecular factors and mechanisms underlying this interaction are almost unknown. The aim of the present study was to evaluate the effects of amendments and bacteria, both alone and in combination, on *Arabidopsis thaliana* grown on arsenic and lead polluted soils. To accomplish this aim, a pot experiment was performed testing the effect of biochar and/or autochthonous metal(loid) resistant *Bacillus* isolates on physicochemical soil properties and on plant growth and metal(loid) uptake/intake. Furthermore, bioinformatics assisted proteomics was used to understand common and specific mechanisms regulating plant growth and metal(loid) tolerance in tested conditions. Results showed that biochar and/or *Bacillus* induced significant and positive effects on soil properties, increasing pH, C_{tot}, N_{tot} and P_{tot} concentrations and decreasing nutrients (N_{av} and P_{av}), As and Pb availability. Plant growth was also enhanced by addition of biochar and/or bacterial inoculum, reaching the maximum when biochar and microorganism were combined. The deciphering of molecular mechanisms revealed that combination of biochar and bacterial inoculation mitigate *Arabidopsis* growth and defense tradeoff, and underline the great potential of plant-biochar-inoculum synergic application in more effective and large scale-up phytostabilizing systems.

3 Conclusions and future perspectives

Assisted phytoremediation is a promising technology for the remediation of metal(loid) contaminated soils, and may become an even more reliable and efficient approach through insights provided by both applied and basic research studies. In recent years, several studies have defined the many factors (*e.g.*, environmental conditions, soil composition, contaminant characteristics, amendment type, and rhizosphere activities) that influence plant performance in assisted phytoremediation processes of metal(loid) polluted areas. However, further research is needed to obtain more information to be used in improving these phytoremediation activities. In such context, the objectives of this thesis were: (i) evaluate the potential of different phytoremediation approaches assisted by soil amendments and/or microorganisms, and (ii) gain a deeper understanding of the biological processes underlying metal(loid)-plant-amendment-microbe complex interactions in metal(loid) contaminated soils. In order to achieve these objectives, three studies were conducted.

In study I, three different *Salicaceae* species (*Populus euramericana* clone I45/51, *Salix purpurea* and *Salix viminalis*), and two diverse application modalities of the biochar amendment (biochar added on all the soil surface and biochar addition on the soil upper layer) were used in the assisted phytoremediation of an arsenic polluted soil. This study was performed under environmental condition, and two of the main goals were to select the best plant species and understand the best strategy to apply amendments for effective *in-situ* assisted phytoremediation approaches of soils polluted by high amounts of arsenic. Results of this first research work, clearly, indicated that the addition of amendment improved soil physicochemical characteristics, particularly when biochar was applied on all the soil surface. However, even if the amendment ameliorated the contaminated site properties, biochar had negative effect on arsenic stabilization into soil and on plant growth. Indeed, to stabilise arsenic in the soil, another soil amendment such as iron sulphate should be used in combination with biochar. Nevertheless, in this research, the introduction of iron sulphate after eleven months from the start of the experiment did not lead to positive effect. Results also showed that, without the biochar addition to the soil, among the three plant species, *Salix purpurea* was the best candidate to use in an arsenic phytostabilization program. Considering together, this evidence demonstrated that the success of a phytoremediation technique depends on the level at which amendment is incorporated to soil, amendment characteristics and plant species. Moreover, the results of the study provided important information on how to apply a soil amendment in assisted phytoremediation strategies to be used for *in-situ* treatment of contaminated soils. Indeed, this experiment highlights that it is important to apply a soil amendment deeper into the soil and not only on the surface when using plant species with a wide and deep root system. In this way, soil amendments would be more effective to improve the characteristics of polluted areas down to the deepest soil layers, improve the growth of plants used in phytoremediation and, consequently, enhance the efficiency of metal(loid) remediation processes.

In research II, the effect of four different organic amendments (bamboo biochar, oak biochar, steam activated carbon, and acidic activated carbon) combined with red mud was evaluated on characteristics of an arsenic and lead polluted soil, and on growth and metal(loid) phytostabilization ability of *Trifolium repens*. This second study was carried out under controlled condition, and data of the experiment indicated the importance to use an amendment combination when a soil is multi-contaminated by metal(loid)s with different characteristics. Moreover, results also showed the need to utilize several amendment application rates on the base of soil properties, contaminant concentrations, and plant species. The study also showed the suitable use of *Trifolium repens* as a good stabilizer plant for metal(loid)s as arsenic and lead which were stored in plant roots. Therefore, the results of this study could be used to develop successful *in-situ* assisted phytoremediation programs for arsenic and lead contaminated sites using a cover with white clover.

In order to understand the molecular mechanisms underlying plant capacities to growth in a polluted soil and tolerate high metal(loid) concentrations, in paper III, a bioinformatics-assisted proteomics analysis was performed in a phytoremediation study based on plant-amendment-bacteria synergy. For this work, a basic research was conducted on the *Arabidopsis thaliana* model plant grown under controlled conditions.

The data obtained from this study indicated that inoculation with specific contaminant tolerant and plant growth promoting bacteria, belonging to *Bacillus* genus, was effective in enhancing phytoremediation, especially when the bacteria inoculation occurred together with the biochar amendment addition to the arsenic and lead contaminated soil. Furthermore, results suggested that, in a polluted site, plants use all the energy obtained from biogenesis processes to counteract with metal(loid) stress and be able to growth. Interestingly, bioinformatics-assisted proteomics data highlighted that only the combined use of biochar-amendment and bacteria inoculum led to a correct balance of processes related to plant growth and response to metal(loid) stress. Conversely, the addition of amendment or bacteria alone seemed not to be sufficient to guarantee plant growth-defence tradeoffs, thus, their beneficial effect could be not maintained over-time. Indeed, although on one hand, biochar had the ability to increase plant growth by providing nutrients and reducing metal(loid) availability, on the other hand it seemed to induce dysfunction of plant defence mechanisms and increase their susceptibility to pathogen attack.

In contrast, on one side, the *Bacillus* inoculation improved plant growth by releasing plant growth-promoting substances but, on the other side, it did not appear to be effective in reducing metal(loid) availability and mitigating oxidative stress in plants. The positive results of this research on the growth and response of *Arabidopsis* in metal(loid) contaminated soil lay the foundation for the use of plant-biochar-microorganism combinations in more effective and large-scale assisted phytoremediation systems. However, future research studies using different application rates and types of biochar, plant species and bacterial strains are needed to determine which signals trigger the correct trade-off between growth and defence system of plants in a polluted soil.

Taken together, the results of the applied studies on *Salicaceae* and *Trifolium repens*, combined with data from the proteomics approach on *Arabidopsis thaliana*, helped to characterise the interactions between metal(loid)s, plants, amendments and microorganisms in polluted soils.

In a prospective view for this doctoral research, further efforts may be proposed to maximise the management of metal(loid) contaminated lands, and develop more effective assisted phytoremediation strategies. The potential of biological systems in the assisted remediation of contaminated sites can be exploited and enhanced by carefully selecting plant species, soil amendments and microorganisms. In addition, further genetic and "omics" studies could lead to a broader understanding of the biological processes underlying the complex interactions involved in assisted phytoremediation techniques. Through genetic engineering, this knowledge would help in the design of next-generation plant and microbial species useful for improvement of plant growth and specific suppression of their susceptibility to abiotic and biotic stresses.

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