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Proceeding Paper

Bioinformatics-Assisted Proteomics of Metal(loid) Tolerance in *Arabidopsis* †

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Abstract: In recent years, the restoration of metal(loid) polluted soils through the combined use of plants-amendments-microorganisms is a strategy that is receiving great attention. However, the molecular processes underlying this synergy are not fully understood. Thus, the aim of this work was to provide insight into the biological mechanisms used by *Arabidopsis thaliana* to grow in soil contaminated by arsenic and lead and amended with biochar and/or *Bacillus* sp. inoculum. To accomplish this goal, a pot experiment was set up and the effects of the biochar amendment and the bacterial isolate were evaluated, both alone and in combination. The effects of the plant-biochar-bacteria synergy were assessed on soil physicochemical characteristics, plant growth and ability to stabilize or accumulate metal(loid)s. In addition, a bioinformatics-assisted proteomics approach was used to understand the molecular processes underlying *A. thaliana* growth in the different tested conditions. Results showed that the use of biochar and/or *Bacillus* inoculum resulted in improvements in soil properties and plant growth. Bioinformatics-assisted proteomic analysis showed that, on the one hand, the use of biochar alone led to an over-representation of proteins involved in nutrient metabolism providing plants with essential nutrients for growth. However, biochar alone induced plant defense mechanism dysfunction and increased susceptibility to pathogen attack. On the other hand, the use of bacterial inoculation helped plants to grow thanks to the activation of molecular pathways involved in the defense against biotic stress. Only the combined use of biochar and bacteria ensured the correct balance between molecular processes associated with growth and metal(loid) stress response in *Arabidopsis* plants.

Keywords: *Arabidopsis thaliana*; *Bacillus* sp.; biochar; bioinformatics; metal(loid)s; proteomics

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1. Introduction

Trace metal(loid)s are biologically essential for organisms to maintain normal life activities [1]. However, excessive metal(loid) accumulation in soils is harmful to animal life, human health, and plants. Consequently, the remediation of metal(loid) polluted sites became mandatory to reduce the associated risks.

Several technologies exist to remediate metal(loid) contaminated soils, and in recent years, a remediation strategy that uses vegetation, associated microbiota and soil amendments is proving to be a promising option to manage lands polluted by metal(loid)s [2].

Plants utilize a series of defense mechanisms to control metal(loid) uptake, accumulation, and translocation such as metal(loid) exclusion, sequestration, compartmentalization in specific cell organelles (e.g., vacuoles), and inactivation by exudation of organic ligands. Diverse specific proteins take part in these metal(loid) detoxification mechanisms. Indeed, apart from the plasma membrane exclusion method, a common way to protect plant cell from the adverse effects of metal(loid)s includes synthesis of membrane transporters and thiol-containing chelating compounds [3]. Furthermore, increased abundance of defense proteins for effective reactive oxygen species (ROS) scavenging and molecular chaperones for re-establishing normal protein conformation help metal(loid) stressed plants to maintain redox homeostasis [4]. Modulations of vital metabolic pathways—such as photosynthesis and mitochondrial respiration—further help the stressed plant to produce more reducing power to compensate high-energy demand of metal(loid) challenged cells [5].

Nevertheless, when the contaminated sites are heavily polluted, severe toxicity or competition with key micronutrients leads to a plant growth reduction. In this context, plant efficiency to tolerate metal(loid) toxicity can be enhanced through two main techniques: (i) amelioration of soil characteristics and plant growth through amendment use, and (ii) enforcement of the plant activity with soil microorganisms [6].

In the last few decades, widely used as a soil amendment is biochar which is a carbonaceous product obtained through the pyrolysis of biomass (e.g., crop residues, dairy manure, and wood) in the partial or total absence of oxygen. 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 [7].

Microorganisms also bring many benefits since they perform multiple functions, such as improvement of soil quality, and enhancement of plant growth and plant capacity to tolerate, remove or stabilize metal(loid)s [2].

In consideration of the facts, 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 restore polluted lands [8]. However, 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 [2].

In recent times, "omics" techniques are being exploited to identify plant molecular strategies of metal(loid) stress tolerance. As proteins play a key role in plant stress response, proteomics studies provide a finer picture of protein networks and metabolic pathways primarily involved in cellular detoxification and tolerance mechanisms against metal(loid) toxicity [9]. Special emphasis is given to highlight the role of metal(loid) stress-related proteins engage in metal(loid) ions sequestration, antioxidant defense system, and primary metabolism for deeper understanding of pathways involved in detoxification of metal(loid) ions within plant cells [3].

Arabidopsis thaliana proteomics has taught great lessons on different aspects of plant growth, development, and physiology. Indeed, more than a decade after the sequencing of its genome, *Arabidopsis thaliana* still stands the most widely used model system in plant biology [10]. Without doubt our understanding of basic principles of plant biology would not have been this advanced if it were not for knowledge gained using *Arabidopsis* as a model system. *Arabidopsis* is also greatly used in studies aimed at understanding metal(loid) stress tolerance and adaptation [11].

In this context, in the present study, the effects of metal(loid)s, biochar, and bacteria (both alone and in combination) were evaluated on *Arabidopsis thaliana* plant growth and development on an arsenic and lead polluted soil. Moreover, a bioinformatics-assisted proteomics analysis was of great importance for a better understanding of the tolerance mechanisms by which plants respond to metal(loid) stress.

2. Materials and Methods

2.1. Contaminated Soil, Biochar and Bacillus sp.

The contaminated soil used for the present study was a technosol derived from a former silver-lead mine extraction site located in Pontgibaud (France). The site is contaminated by arsenic (539.06 ± 0.01 mg·kg^{-1}) and lead (11453.63 ± 0.18 mg·kg^{-1}) [12].

The biochar used was provided by La Carbonerie (Crissey, France) and derives from the slow pyrolysis of charm wafers and chips, beech and oak. The main physicochemical properties of the biochar were determined in previous works [13,14].

An autochthonous metal(loid) resistant bacteria strain was selected from Pontgibaud technosol, and successively characterized as detailed in [15]. The 16S rRNA gene sequencing led to identify the microorganism as *Bacillus* sp. [16].

2.2. Pot Experiment and Soil Pore Water and Plant Analysis

Four conditions were tested, and four pots were prepared for each condition: non amended Pontgibaud technosol (Po); Po + 2.5% biochar (PoB); Po + Bacillus sp. inoculum (PoI); and Po + 2.5% biochar + Bacillus sp. inoculum (PoBI). 50 seeds of Arabidopsis thaliana were placed to germinate in each pot and plants were grown for 32 days in a growth chamber under controlled conditions.

At the end of the experiment, soil pore water (SPW) samples were collected as detailed in [16] and used to measure pH and As and Pb concentrations.

After the 32 days of plant growth, dry weight (DW) was determined for the different *A. thaliana* organs (roots, stems and leaves). As and Pb concentrations in the plant organs (roots, stems and leaves) were also measured.

2.3. Proteomics and Bioinformatics Analysis

Total proteins were extracted from A. thaliana leaves and separated by two-dimensional gel electrophoresis (2-DE). Protein spots of interest were excised from gels and subjected to mass spectrometry analysis. Differentially represented proteins among treatments were identified together with the functional context in which each protein operates and, thus, with the biological processes commonly and specifically activated by the different conditions. The proteomics and bioinformatics analysis details are reported in [16].

2.4. Statistical Analysis

The statistical tests were performed using R software Version 3.4.3 (R Development Core Team, 2017). The normality and homogeneity of variance were assessed using Shapiro-Wilk and Bartlett tests, respectively. The means were compared using ANOVA or Kruskal-Wallis tests, according to normality, followed by a post hoc test to compare modalities between each other.

3. Results and Discussion

3.1. Soil Pore Water and Plant Analysis

Soil pore water (SPW) analysis showed that Pontgibaud soil presented an acidic pH, which was increased in the two treatments containing biochar (PoB, PoBI). SPW As concentration decreased to undetectable levels in both PoB and PoBI soils. Regarding SPW Pb concentration, PoB and PoBI led to decreases compared to Po (Figure 1a).

These results showed that the addition of biochar and/or *Bacillus* to As and Pb contaminated soil induced significant and positive effects on soil properties. Many studies demonstrated that biochar induces an increase of soil pH and several mechanisms could explain such effect of biochar on pH: (i) the alkalinity of the biochar; (ii) the dissolution of basic cations and carbonates that can neutralize the soil acidity; (iii) surface functional groups negatively charged able to bind H$^+$ ions from the soil [17]. The treatments with biochar and/or *Bacillus* also had the capability to reduce As and Pb availability in SPW.

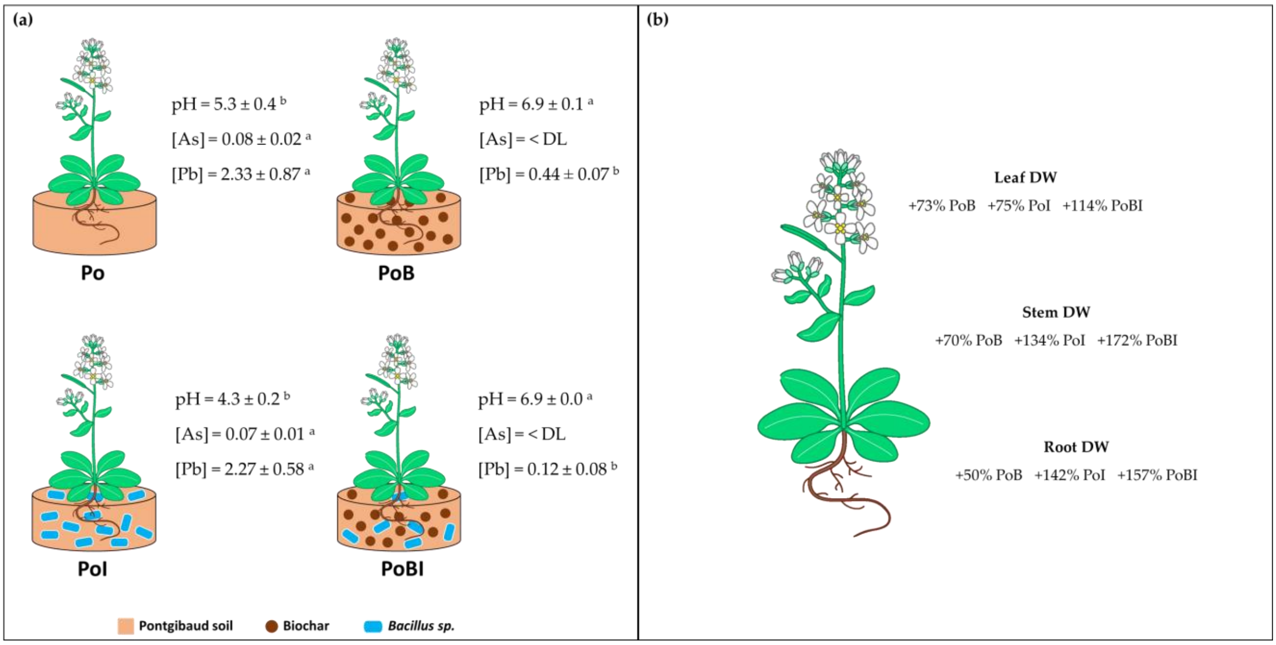

Figure 1. Soil pore water pH, and arsenic [As] and lead [Pb] concentration determined in the four different treatments (Po, PoB, PoI, PoBI). Different letters indicate significant difference ($p < 0.05$) ($n = 4 \pm$ SE). <DL: under detection limit (**a**). Percent increase in leaf, stem, and root dry weight (DW) in PoB, PoI and PoBI compared with the Po treatment ($n = 20 \pm$ SE) (**b**).

The improvement of soil physicochemical properties by the addition of biochar and/or Bacillus to the Pontgibaud polluted soil led also to significant and positive effects on the A. thaliana plant growth. In particular, an amelioration of plant biomass allocation was observed, with the maximum extent in plant grown on the substrate added with the biochar-inoculum combination (Figure 1b). Indeed, leaf, stem, and root dry weight (DW) increased in all the amended soils (PoB, PoI and PoBI) but they reached the highest values in PoBI (Figure 1b). It was understandable that the combined use of amendment-bacteria led to an enhanced plant growth compared to the use of amendment or bacteria alone. Indeed, as demonstrated in previous research [20], biochar provided more bioavailable nutrients (e.g., organic carbon, phosphorous) to bacterial growth and, in turn, microorganisms released plant growth promoting (PGP) metabolites into the soil, which improved plant growth, development and tolerance under toxic metal(loid) conditions.

Analysis of meta(loid) accumulation in different plant organs showed that both As and Pb concentrations were higher in roots than stems and leaves (Figure 2). This containment of metal(loid)s in roots was also observed in other studies and it is considered an avoidance mechanism against metal(loid) toxicity. Metal(loid) containment in roots could be mainly ascribed to the binding cell wall capacity as well as metal(loid) sequestration into the vacuoles [21].

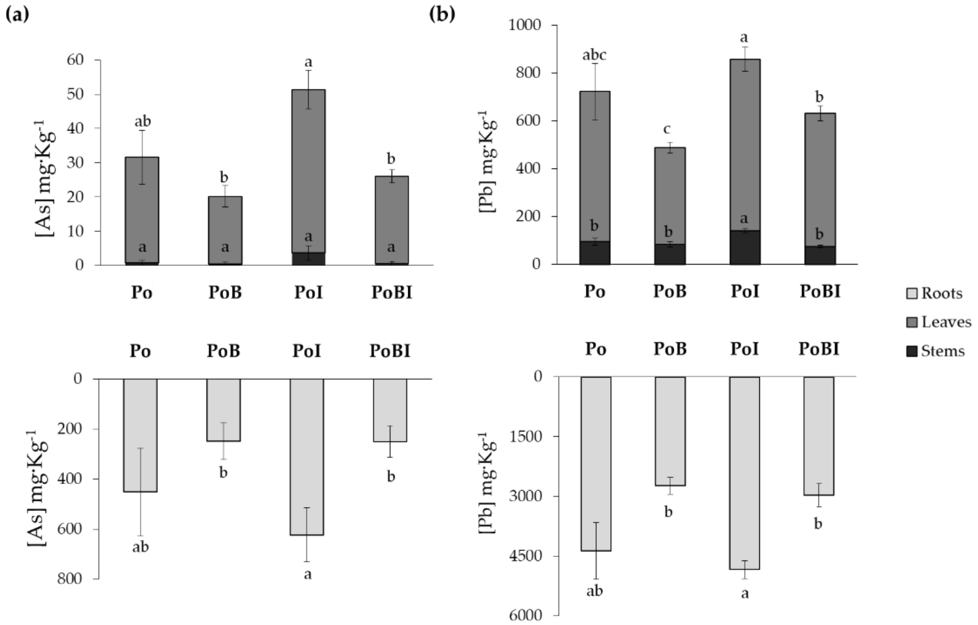

Figure 2. Metal(loid) content in the different plant organs of A. thaliana plants grown on the four different treatments (Po, PoB, PoI and PoBI). Arsenic [As] (Panel **a**) and lead [Pb] (Panel **b**) concentration. Different letters indicate significant difference ($p < 0.05$) ($n = 4 \pm$ SE).

3.2. Bioinformatics-Assisted Proteomics Analysis

The understanding of the mechanisms by which biochar and/or Bacillus inoculum may influence A. thaliana growth and tolerance of metal(loid) stress came from the bioinformatics-assisted proteomics approach of this study. The latter was able to identify a variable number of Gene Ontology (GO) biological process terms commonly or specifically characterizing Po, PoB, PoI and PoBI cluster-related subnetworks. In detail, the GO terms commonly characterizing all cluster-related subnetworks were mainly related to energy and primary metabolism and, thus, to biological processes important to guarantee plant functionality and development in abiotic stress condition [22]. The GO terms specifically characterizing cluster-related subnetworks, conversely, represented different adaptive aspects of A. thaliana able to assure all plant cell functions and to overcome the metal(loid) injury.

Some important biological processes found in this study were primarily related to transmembrane transport, protein synthesis, proteasome activity, and oxidative stress-induced response (Figure 3). Plasma membrane is the primary environmental barrier for a plant cell that mediates the exchange of information and materials between the cell interior and the extracellular environment [23]. Thus, plasma membrane also plays an essential role in metal(loid) tolerance and detoxification providing a range of membrane-bound transporters able to remove toxic agents from the cytosol either by catalyzing their export or sequestration [23]. Moreover, as reported in a previous study [24], proteins are key target of metal(loid)s, which can cause denaturation and aggregation of nascent proteins interfering with their folding and refolding. Thus, it was reasonable to hypothesize that A. thaliana plant, to overcome metal(loid) toxicity, responded by over-representing proteins related to degradation pathways, including proteasome (Figure 3). According to other authors [4,5], our results also showed that plants of Arabidopsis tolerated the metal(loid) injury with the activation of molecular processes able in preventing reactive-oxygen species (ROS) metal(loid)-induced accumulation.

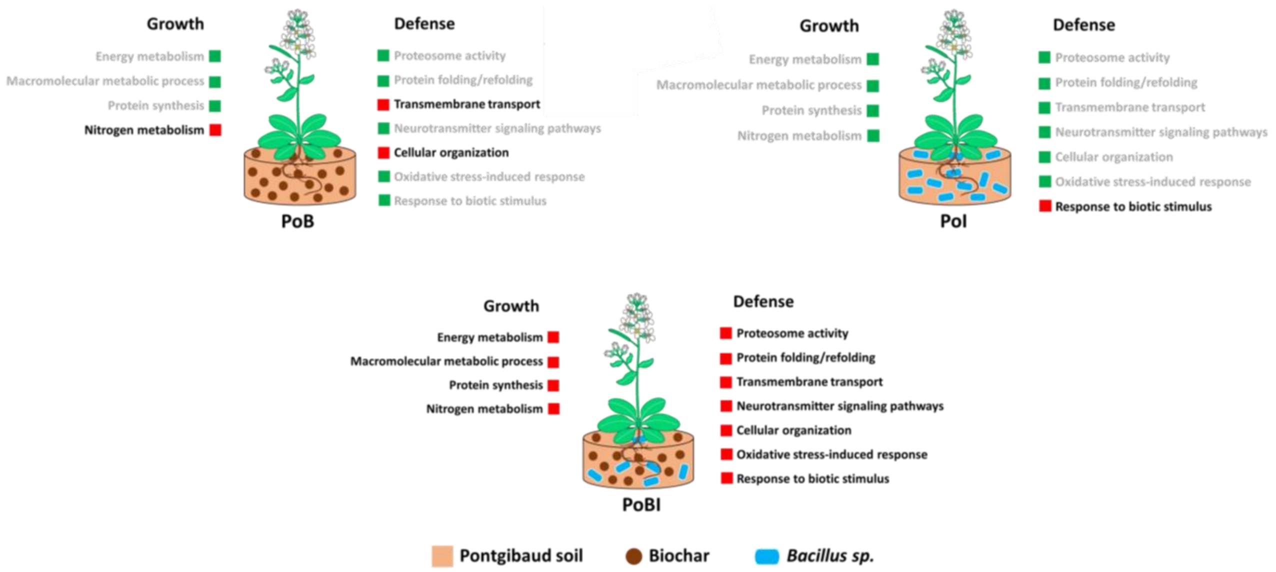

Figure 3. Scheme summarizing the effect of biochar and/or Bacillus on A. thaliana growth/defense. Red and green box are used to indicate over- or under-representation, respectively, of protein abundance profiles belonging to growth and defense GO terms biological processes in the PoB, PoI and PoBI treatments.

In order to understand the differences between plants grown in the treatments with the addition of biochar and/or bacteria (PoB, PoI and PoBI) compared to those grown in the contaminated soil (Po), a distinction of biological processes into mechanisms useful to A. thaliana in growth and stress defense was made (Figure 3). Firstly, these data showed that in PoB treatment, the A. thaliana plant growth enhancement was associated with the ability of biochar to supply nutrients such as nitrogen, considering the fact that there was an over-representation of protein abundance profiles (PAP) belonging to molecular pathways associated with nitrogen metabolism. However, the PoB treatment was characterized by under-representation of PAPs related to molecular mechanisms of defense toward abiotic and biotic stresses (Figure 3). Indeed, biochar alone induced dysfunction of the plant defense machinery and increased susceptibility to pathogen attack [25]. Similarly, the use of bacterial inoculum alone did not have the best effect on plant growth and tolerance to metal(loid) stress as visible by the fact that the PoI treatment was characterized by under-representation of PAPs associated with those biological processes that act in plant defense against environmental stresses (Figure 3). Lastly, bioinformatics-assisted proteomics analysis confirmed and explained the results obtained through plant analyses in that, the treatment with the combined addition of biochar and Bacillus inoculum (PoBI) was the best one for A. thaliana growth and development. Indeed, PoBI condition was characterized by an over-representation of both PAPs associated with plant growth and defense mechanisms (Figure 3). This indicated that the best plant growth was recorded in PoBI because there was a correct balance between the biological processes related to the growth/development of A. thaliana and those related to the response to metal(loid) stress.

4. Conclusions

In this study, Arabidopsis thaliana plants were grown on an arsenic and lead polluted soil (Po) supplemented with biochar and bacteria (both alone and in combination). The results of physicochemical analysis showed that the use of biochar soil amendment and Bacillus sp. inoculation improved soil properties by increasing pH and reducing phytoavailable metal(loid) concentrations. Moreover, the results of our experiment highlighted that these improvements were maximum when biochar and bacteria were used in combination. Analyses conducted on the model plant A. thaliana revealed how plants stabilized metal(loid)s at the root level using defense mechanisms to cope with the toxicity of arsenic and lead. In addition, the results of the bioinformatics-assisted proteomics approach allowed us to understand the molecular mechanisms which were used by Arabidopsis, in the different tested conditions, to overcome metal(loid) injury. On the one hand, in the treatment with the biochar alone (PoB), plants grew better than in the contaminated soil (Po) because the biochar was able to increase the availability of important nutrients such as nitrogen. Indeed, in the PoB soil there was an over-representation of protein abundance profiles (PAPs) associated with nitrogen metabolism and an over-representation of PAPs associated with transmembrane transport. Transmembrane proteins may have been used to remove metal(loid) ions from within the plant cell. On the other hand, the use of the bacterial inoculum (PoI) allowed plant growth on the contaminated soil due mainly to the activation of proteins involved in stress defense mechanisms in plants. However, growth and development of Arabidopsis were better when biochar and bacteria were added in combination to the soil (PoBI). In PoBI treatment, there was an over-representation of PAPs associated with pathways related to primary metabolism of energy production, protein synthesis, nutrient metabolism, and all those molecular mechanisms involved in the response to abiotic and biotic stresses (e.g., proteasome activity, protein folding and refolding, response to oxidative stress).

In conclusion, our study helped to characterize the interactions among metal(loid)s, plants, amendments, and microorganisms in polluted soils. Moreover, the research showed that the combined use of the biochar amendment and Bacillus inoculum resulted in a proper balance of processes related to Arabidopsis thaliana growth and metal(loid) stress response in an arsenic and lead polluted soil. Thus, the results obtained emphasize that the plant-amendment-bacteria synergy has great potential to be applied in remediation strategies of metal(loid) contaminated sites on a larger scale. Finally, further genetic and "omics" studies should be conducted to design, through genetic engineering, next-generation plant and microbial species useful for improving plant growth and specifically suppressing their susceptibility to abiotic and biotic stresses.

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