Effect of *Salix atrocinerea* root-associated bacteria on the phytoremediation of arsenic-polluted soils

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"Efecto de bacterias asociadas a las raíces de *Salix atrocinerea* en la fitorremediación de suelos contaminados con arsénico"

"Effect of *Salix atrocinerea* root-associated bacteria on the phytoremediation of arsenic-polluted soils"

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RESUMEN (en español)

En toda Europa, los suelos están muy amenazados por la contaminación, siendo los elementos traza (ET) los contaminantes químicos más peligrosos. Entre éstos, el arsénico (As), sin función biológica conocida, es absorbido por las plantas y causa daños severos en la mayoría de las especies. Para evitar la toxicidad de los ET, algunas plantas presentan diferentes mecanismos de tolerancia que les permiten crecer en áreas altamente contaminadas, una propiedad que puede ser explotada en la remediación de suelos contaminados. Uno de los inconvenientes de esta fitorremediación es el largo tiempo requerido para una descontaminación efectiva. Sin embargo, este proceso puede acelerarse mediante el uso de especies leñosas productoras de gran biomasa y que crecen naturalmente en las áreas contaminadas; e incluso mejorarse a través del uso combinado de los microbios asociados a sus raíces. En el terreno de estudio, el As es el contaminante más presente y supera en 20 veces el valor máximo de referencia asignado para el desarrollo de actividades industriales. Pese a que la mayor parte de este As está en forma de arseniato (As V), que es menos móvil que el arsenito (As III), se ha observado que algunas bacterias pueden reducir As V a As III, lo que aumenta su disponibilidad y mejora la absorción del metaloide por parte de la planta, reduciendo de este modo el tiempo necesario para la descontaminación.

A través de medios de cultivo e independientes del mismo, se identificaron y caracterizaron las comunidades microbianas asociadas a las raíces de *S. atrocinerea*. Aquellas cepas con mayor potencial para mejorar la eficacia de la fitorremediación fueron seleccionadas según su tolerancia al As, la capacidad para reducir As y la presencia fenotípica de propiedades que potencian el crecimiento vegetal. En base a esto, la bacteria gram negativa *Pantoea* sp. y la gram positiva *Rhodococcus erythropolis* fueron seleccionadas para experimentos de bioaumentación.

En un primer ensayo en hidroponía en presencia de As con *S. atrocinerea* se evaluó la tolerancia de la planta y la acumulación del metaloide en sus tejidos. Las plantas de sauce mostraron la regulación de genes implicados en respuestas al estrés, reducción de As V, transportadores de nutrientes, síntesis de glutatión y en el almacenamiento del As en las vacuolas y, que coincidió con una rápida presencia y acumulación de As III en raíces, una alteración del perfil nutricional y la síntesis *de novo* de compuestos tiólicos no proteicos. Sugiriendo, por tanto, que esta respuesta coordinada de quelación y

disminución de la toxicidad de As permite a la planta restablecer su homeostasis y continuar con su desarrollo.

Posteriormente, tras la inoculación *in vitro* de *S. atrocinerea* con *Pantoea* sp. o *R. erytrhopolis*, se observó que ante la presencia de As la inoculación bacteriana en plantas constituye un mecanismo sinérgico que afecta a la toxicidad del metaloide al alterar su especiación, movilidad y su posterior acumulación en los tejidos vegetales. Influyendo la inoculación bacteriana, de esta forma, en la respuesta de los mecanismos de adaptación al As de la planta.

En maceta, la reducida acumulación de los ET obtenida en plantas inoculadas con *Pantoea* sp. o *R. erythropolis* en un suelo contaminado ocasionó a una mayor biomasa en comparación con las plantas no inoculadas, que a largo plazo puede aumentar la fitoextracción de los ET. Sin embargo, en el experimento de campo, debido a diferentes factores bióticos y abióticos, la inoculación bacteriana resultó en una mayor acumulación de As y Pb en la planta, y por lo tanto en una mayor fitoextracción, junto con una elevada translocación de Zn. Mientras que en plantas inoculadas con *Pantoea* sp. esto ocurrió por una mayor acumulación de ET que originó una reducción de biomasa, en *R. erhytrhopolis* se obtuvo por medio de una menor acumulación de ET pero con una biomasa mayor que en plantas no inoculadas.

En conjunto, se observó que los resultados obtenidos en el laboratorio no siempre pueden extrapolarse al campo y que los ensayos de campo son esenciales para lograr una fitorremediación exitosa. No obstante, los estudios de laboratorio han proporcionado un considerable conocimiento acerca de la tolerancia a ET de la planta y las bacterias, así como de su interacción. Además, la posible utilidad de las bacterias aisladas de *S. atrocinerea* puede cruzar la barrera entre especies tal y como se observó a través de la inoculación de *Pantoea* sp. AV62 a *Arabidopsis thaliana*. Lo cual proporciona una mayor información sobre los mecanismos implicados en la interacción bacteria-planta y que es importante en el desarrollo de estrategias específicas de remediación de ET por medio del binomio bacteria-planta.

RESUMEN (en Inglés)

All around Europe soils are very threatened by contamination, being trace elements (TEs) the most dangerous chemical pollutants. Among these, arsenic (As), despite having no biological function, is taken up by plants, causing severe harm in most species. To avoid TE toxicity, some plants present different tolerance mechanisms that allow them to grow in highly polluted areas, a property that can be exploited in the remediation of contaminated soils. One of the drawbacks of this phytoremediation is the long time required for effective decontamination. However, this process can be accelerated by the use of high biomass-producing woody species naturally growing in the polluted areas in combination with the enhancement of their root-associated microbes. In the soil of study, As is the most present pollutant and exceeds by 20 times its assigned maximum reference value for industrial activities. However, most of it is in the form of arsenate (As V) which is less mobile that arsenite (As III), but it has been observed that some bacteria are able to reduce As V to As III, which increases its availability and enhances the plant uptake of the metalloid while reducing the time needed for decontamination.

By culture-dependent and culture-independent approaches, the microbial communities associated to the roots of *S. atrocinerea* were identified and characterized. The most promising strains to improve phytoremediation efficiency were selected based

on As tolerance, As reduction and presence of plant-growth-promoting (PGP) traits. According to this, the gram-negative *Pantoea* sp. and the gram-positive *Rhodococcus erythropolis* were selected for further bioaugmentation experiments.

On a first hydroponic assay with *S. atrocinerea* under As V presence to test plant tolerance and accumulation of the metalloid*,* willow plants showed a transcriptional regulation of genes involved in stress responses, As V reduction, nutrient transporters, glutathione synthesis and sequestration of As into the vacuoles, which coincided with a rapid As III presence and accumulation in root tissues, altered nutrient profile and *de novo* synthesis of non-protein thiolic compounds. Suggesting that this coordinated response helps the plant to re-establish its homeostasis and progress with its development stages by complexing As and limiting its toxicity.

Later on, when *S. atrocinerea* was inoculated *in vitro* with *Pantoea* sp. or *R. erythropolis*, it was observed that plant inoculation with bacteria constitutes a synergistic mechanism that alters the toxicity of arsenic by changing its speciation state, mobility and its subsequent accumulation in plant tissues. Consequently, bacteria inoculation affects the response of the plant's adaptive mechanisms under As presence.

In pot, the reduced TE accumulation obtained in inoculated plants with *Pantoea* sp. or *R. erythropolis* in a polluted soil led to a higher biomass in comparison to non-inoculated plants, that in the long-term can increase the phytoextraction of TEs. However, in field experiment, due to the different biotic and abiotic factors involved, bacterial inoculation resulted into a higher As and Pb accumulation in the plant, and therefore a greater phytoextraction, together with a markedly higher Zn translocation. While in plants inoculated with *Pantoea* sp. this was achieved by a higher accumulation of TEs with a biomass decrease, in *R. erythropolis* it was obtained by a lower accumulation but higher biomass than in non-inoculated plants.

Altogether, it was observed how the results obtained in the laboratory cannot always be extrapolated to the field and how these field assays are essential for a successful phytoremediation. However, laboratory studies have provided a considerable knowledge about As tolerance by plant and bacteria and their interaction. Furthermore, the potential application of the bacteria isolated from *S. atrocinerea* can cross the interspecies barrier as seen by inoculation of *Pantoea* sp. AV62, a root-associated bacteria of *S. atrocinerea,* to *Arabidopsis thaliana*, which provides more information about the mechanisms implied in bacteria-plant interaction and such information can be derived to tailor specific pollutant-bacteria-plant remediation strategies.

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Plants are thermodynamically open systems that exchange matter and energy with the environment that surrounds them. Those were the words that I heard from a former lecturer in *Plant Physiology* to avoid that students underestimate the study of plants in comparison with that of other "more attractive" organisms, and those words did not only awake my interest about the topic but, in a similar way, I think that definition describes my experience during these past 5 years, where *plants* could be easily replaced with *PhD students*. Therefore, I'd like to thank all the people that put their energy and matter into this research. In first place, to the group of Tecnología, biotecnología y geoquímica ambiental (BIOGEOAMB) and to the Centrum voor milieukunde UHasselt (CMK), especially my promotors for their research leadership. To all my doctoral colleagues, lab personal, professors and students, thanks for the exquisite support in this adventure. Thanks to the Ministry of Education, Culture and Sport and to that of Employment and Social Security for the economic support. Finally, friends and family, thanks for the moral and physical support and, the most important, to help in putting things into perspective.

Gracias,

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Brief statement

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ABBREVIATIONS

ABC	ATP-Binding Cassette transporter
ANOVA	Analysis of variance
As III	Arsenite
As V	Arsenate
As V + P	*In vitro* plants exposed to As V and inoculated with *Pantoea* sp.
As V + R	*In vitro* plants exposed to As V and inoculated with *Rhodococcus erythropolis*
BCF	Bioconcentration factor
BCR	European Community Bureau of Reference
bp	base pairs
C	*S. atrocinerea* plants grown on control substrate in pot
CDC25	Tyrosine phosphatase (arsenate reductase)
Chl	Chlorophyll
CP	*S. atrocinerea* plants grown in control substrate in pot and inoculated with *Pantoea* sp.
CR	*S. atrocinerea* plants grown in control substrate in pot and inoculated with *R. erythropolis*
CT	Condensed tannins
CTAB	Hexadeyltrimethylammonium bromide
Cys	Cysteine
DTNB	5,5-dithiobis-(2-nitrobenzoic acid)
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
F	*S. atrocinerea* plants grown in a polluted field
FP	*S. atrocinerea* plants grown in a polluted field and inoculated with *Pantoea* sp.
FR	*S. atrocinerea* plants grown in a polluted field and inoculated with *R. erythropolis*
FW	Fresh weight
GR	Glutathione reductase
GS	Glutathione synthase
GSH	Glutathione
GST	Glutathione-S-transferase
HPLC	High performance liquid chromatography
HSD	Tuckey's least significant difference test
ICP-MS	Inductively coupled plasma-mass spectrometry
KEGG	Kyoto encyclopedia of genes and genomes
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
M	*S. atrocinerea* plants grown on polluted soil in pot
MDA	Malondialdehyde
MP	*S. atrocinerea* plants grown on polluted soil in pot and inoculated with *Pantoea* sp.
MR	*S. atrocinerea* plants grown on polluted soil in pot and inoculated with *R. erythropolis*
MS	Murashige and Skoog
NCBI	National Center for Biotechnology Information
NPT	Non-protein thiol
NSTI	Nearest Sequenced Taxon Index
PC	Phytochelatin
PCA	Principal component analysis
PCoA	Principal coordinate analysis

PGP	Plant-growth-promoting
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PVP	Polyvinylpyrrolidinone
PVPP	Polyvinyl-polypyrrolidinone
QIIME	Quantitative Insights into Microbial Ecology
ROS	Reactive oxygen species
RT-qPCR	Reverse Transcription quantitative PCR
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TE	Trace element
TF	Translocation factor
TFA	Trifluoroacetic acid
TP	Total phenols
USDA	United states department of agriculture
VAPs	Vertical agar plates
WPM	Woody plant medium

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SUMMARY

I. INTRODUCTION

Despite soils are the base and support for life and are a non-renewable resource which perform many functions and deliver services vital to human activities and to ecosystem survival, they are very threatened by contamination. One of the main chemical contaminants (referred as the presence of a substance where it is not expected to be or above its background levels) of soils are trace elements (TEs). These chemicals occur usually at concentrations less than 100 mg kg^{-1}, such is the case of cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg), zinc (Zn), boron (B) or arsenic (As), although, as a matter of fact, many of these previous elements are present at concentrations much lower than this, whereas other like iron (Fe) exceed those limits. The origin of TEs in soil may be either natural (lithogenic) or anthropogenic (human-induced). The anthropogenic activities are the major causes of TE accumulation in soils, where inputs are mainly related to industrial activities, which exploit land for mineral extraction, manufacturing, waste disposal and aerial deposition of fumes; as well as agriculture, such as use of wastes, fertilizers, and soil erosion. When these hazardous substances are present in the soil at a level that pose a significant risk to the environment and human health, they are referred as pollutants and they have deleterious implications for the wider environment, including human health. Such is the case of Nitrastur (Langreo, Asturias), one of the main fertilizer factories in Spain for more than fifty years until its closure in 1997, that is among the polluted brownfields identified in the region with high levels of As and Pb.

Although, some TEs such as B, Cu, Fe, manganese (Mn), or Zn, are indispensable for the normal plant growth and development, and therefore are also regarded as essential micronutrients, TEs such as the metals Cd and Hg, and the metalloid As have no demonstrated biological function in plants but are nevertheless taken up and cause severe toxicity in most plant species. After their uptake and transport into the plant tissues, free TEs ions exerts their toxicity through three major mechanisms: 1) Competing (due to chemical similarity) for the same transporters in root cells, which leads to nutritional deficiencies and, once inside the cells, can displace essential metal ions in metalloproteins, interfering with the biological activity of important enzymes and signaling proteins. 2) Binding with high affinity to free thiols or other functional sulfhydryl and carboxyl groups

of important proteins, pigments or enzymes and inhibiting their function. 3) Enhancing the generation of reactive oxygen species (ROS) causing oxidative stress in plant cells.

However, plants present different tolerance mechanisms that imply maintaining low concentrations of free TEs ions in their cells to prevent the damaging effects described above and include a transport limitation through the membrane, exclusion, chemical reduction, chelation by glutathione (GSH), phytochelatins (PCs), metallothioneins (MT), as well as induction of heat shock proteins (HSP). Furthermore, to cope with TEs-induced oxidative stress, plants possess an extensive scavenging system for ROS excess. This include enzymatic (catalase, peroxidases, superoxide dismutase…) and non-enzymatic antioxidants (GSH, some phenolic compounds, proline, carotenoids…).

Consequently, due to the coordinated effect of the above-mentioned mechanisms some plants can grow in heavily polluted areas and this property can be exploited in the remediation of contaminated soils. This process is called phytoremediation and it is proposed an effective green alternative to the expensive physio-chemical remediation technologies. Nevertheless, phytoremediation has also limitations, being the most recognizable that it is usually slower than other conventional remediation techniques. However, the use of woody species, which increase the biomass yield, together with the enhancement of their root-associated microbes can accelerate this process. Moreover, this bioaugmentation process can promote plant health and growth, enhance water and mineral uptake, and even inhibit the growth and development of pathogenic soil microorganisms. Therefore, an integration of microbial bioremediation and phytoremediation is a promising strategy for remediation of pollutant. That in combination with the selection and characterization of native accumulator plants and microbes increases the rate of success in the process. Although several studies have explored the potential of willow species for phytoremediation, little research has been done on their tolerance to pollutants. If this tolerance, together with the relationships that underlie its plant-microbiome selection, is understood, the knowledge obtained will be of great advantage for the design of effective phytoremediation processes.

To go a step further, although it is believed that bacteria colonization is more efficient in their native host, a very interesting point would be to explore the consequences of species cross inoculation, including, for example, the inoculation to other plant species of bacteria strains isolated from willow and with a high potential in phytoremediation. This will no only allow to deepen into the bacteria-plant host interaction but also into broadening the spectrum of this isolated and characterized strains in phytoremediation.

II. BACKGROUND AND OBJECTIVES

So far, most of the phytoremediation-based studies described are focused on study independently the effect that bacterial communities and plants have in the uptake, degradation and accumulation of pollutants. Furthermore, this is usually performed in artificially contaminated soils or substrates with just one pollutant and with model plants. Therefore, successful *in vitro* or controlled laboratory research might not grant effective results the field since a wide range of abiotic and biotic interactions need to be taken into account.

Therefore, the main objective of this dissertation has been to study the behavior of bacteria inoculated and non-inoculated plants growing in different experimental conditions, exposed mainly to As and other pollutants, from *in vitro* culture to field; in order to evaluate their suitability for being used in phytoremediation. For this objective we selected a clone of grey willow (*S. atrocinerea*) able to grow naturally in an multi TE-polluted soil and the growth and TE accumulation of inoculated and non-inoculated plants was studied, as well as the detoxification mechanisms and genetic responses behind arsenic tolerance. For this purpose, five different aims have been established:

1. Identify and characterize the bacterial communities associated to roots of *S. atrocinerea* thriving in a highly polluted brownfield. By making use of computational and traditional culture methods, both non-cultivable and cultivable bacteria populations were studied, and their putative and phenotypic characterizations for tolerance to As and plant-growth-promoting traits were performed.

2. Assess the potential of *S. atrocinerea* to tolerate and accumulate As and study the mechanisms involved in these processes. At different time points, the As accumulation and speciation, analysis of thiolic compounds and genetic responses behind As tolerance were studied.

3. *In vitro* study of the effects of bacteria inoculation on As accumulation and tolerance in willow plants. Previously characterized bacteria were tested on willow plants grown *in vitro* culture and exposed to As. A detailed characterization of the physiology mechanisms behind As accumulation and tolerance was performed.

4. Characterize the physiological responses of inoculated and non-inoculated plants cultured in a polluted industrial soil. Inoculated and non-inoculated plants were grown

in pots with a commercial potting substrate or a polluted industrial soil. A comparison of the plant-bacteria interaction under different substrates is done. In parallel, a six-months experiment was performed in the polluted field. The differences obtained among pot and field related to TE accumulation and tolerance are discussed.

5. Evaluate if the bacteria isolated from *S. atrocinerea* can enhance As tolerance in other plant species. Arsenic accumulation and transcriptional changes in *Arabidopsis thaliana* plants inoculated with *Pantoea* sp. were investigated. Furthermore, the role of bacteria inoculation in As tolerance under a different glutathione (GSH) plant supply is evaluated by the use of wild-type and *cad2-1* (GSH deficient) genotypes.

III. RESULTS AND DISCUSSION

The total concentration for Hg, Zn, Cu, Pb and As in the soil of this study exceeded in 3, 6, 7, 15 and 20 times, respectively, the concentration legislated for an industrial soil. In this dissertation a special focus is given to As, pollutant that in the soil of this study exceeded by far its assigned maximum reference value for industrial activities. It has been demonstrated that the bacteria associated to the roots of *S. atrocinerea*, as well as these plants, have developed several mechanisms to cope with As that enables them to tolerate high environmental concentrations of the metalloid. However, despite the high total As concentration in the soil, the metal fractionation analysis (BCR method) showed that only a small fraction of the total As was potentially available, whereas most of the TEs are entrapped within the crystal structure of minerals in non-mobile forms. Despite the actual available TE concentration does not represent such an environmental risk, the soil still needs to be remediated to bring back its economic value. The low As bioavailability in this soil could be explained by the fact that in aerobic soils, as in the soil of study, most of the As (95 %) is in the form of arsenate (As V) which is less mobile that arsenite (As III). However, it is also known that some bacteria can reduce As V to As III. This property can increase As availability in the soil matrix making it more available for plant uptake while reducing the time needed for decontamination.

The genetic diversity associated with rapid growth and peculiar physiological and biochemical characteristics are important requirements for the selection of genotypes useful for phytoremediation. Therefore, in this experimental work, the previous isolation, propagation and clone characterization of an As-tolerant and high As-accumulator *Salix atrocinerea* as compared to literature, together with the isolation and characterization of its

root-associated bacteria, constitutes a suitable approach to enhance phytoremediation since both plant and bacteria growing in a large historically contaminated area have adapted to TEs exposure and both benefit from that interaction. By culture-dependent and culture-independent approaches, it was observed that soil-microbes-roots interactions shape the bacteria communities, as seen in the different bacteria composition observed at soil, rhizosphere and endosphere compartments. The selection criteria to choose of the most promising strains to improve phytoremediation efficiency in this study was based on As tolerance, As reduction and presence of plant-growth-promoting (PGP) traits. According to this, the gram-negative *Pantoea* sp. and the gram-positive *Rhodococcus erythropolis* were selected for further bioaugmentation experiments.

On a first hydroponic experiment with *S. atrocinerea* to test its tolerance to As, the metalloid concentration employed was based on the available fraction at the experimental field. At the concentration of 18 mg As kg^{-1}, some of the physiological responses that confer As tolerance and grant its accumulation in plant tissues even at toxic concentrations were described. After 30 d of As exposure, *S. atrocinerea* did not show any phytotoxic symptoms and it was capable of accumulating a higher As concentration in its leaves than that present in the culture medium. However, *S. atrocinerea* is also able to thrive in a brownfield under the presence of other TEs at toxic concentrations (e.g. Zn, Pb). Consequently, the high As accumulation in *S. atrocinerea*, together with its great biomass, highlights the potential of willow in phytoremediation of multi-polluted environments. From the tolerance mechanisms, the production and accumulation of phytochelatins (PCs), natural chelators in plants that bind and translocate metal(loid)s towards the cell vacuole were observed in roots and leaves of *S. atrocinerea* as an early response to As exposure, highlighting that synthesis and accumulation of non-protein thiols (NPTs) can be considered a response which helps the plants to re-establish their homeostasis and progress with their stages of development by complexing As and limiting its toxicity, which was in accordance with the transcriptional regulation of As transporters and reductases, glutathione synthase and metallothioneins.

Since plant As uptake relies on the As concentration available in the environment and, more importantly, on the As speciation in the soil or culture medium, when willow plants were inoculated with the selected bacteria, it was observed how the differential speciation of As (up to 10% of As III with *Pantoea* sp. and 90% with *R. erythropolis* as compared to the 4% in non-inoculated treatments) affected the parameters previously mentioned of As accumulation, speciation, synthesis of chelators and gene expression. Whereas *Pantoea* sp., increased the uptake of As by the plant, the high concentration of As III caused by *R.*

erythropolis increased toxicity to a threshold that the plant was not able to cope efficiently with, which further prevented As accumulation. The increase in lipid peroxidation only in As-exposed plants inoculated with *R. erythropolis*, reflects that As toxicity is linked to its speciation, and shows that no apparent membrane damage was observed unless high As III concentrations were present in leaves. Apart from altering gene expression of transcripts related to proteins involved in transport, chemical reduction, complexation and sequestration of As, bacterial inoculation caused an up-regulation of flavonoid-related transcripts, which also coincided with an increase in anthocyanins, condensed tannins and total phenols when compared to non-inoculated plants. This is not surprising since flavonoids have been shown as crucial signaling molecules in plant-bacteria interactions. Besides, this high tannin biosynthesis under bacterial inoculation is interesting since increased leaf tannin concentrations can confer plants an advantage by reducing arthropod predation in field conditions.

Field trials are often exposed to a broad number of variable factors, such as the irregular distribution of soil pH, pollutants, nutrients or water, among others, that can affect the responses of plants to the treatments. In this research, by comparing a pot experiment with a field experiment conducted with the same soil, it was shown how the variation in field conditions offered divergent results in growth and TE accumulation in plants grown on pots or in the field and non-inoculated or inoculated with bacteria. Since bacterial growth increases with raising pH, and, in this study, the fact that the pH in the homogenously mixed TE-polluted soil was higher than the one observed in the field could explain, among other factors, some of the differences found between effects of inoculations in pot and field assays. Besides, in the polluted soil of this study, it was observed how the *S. atrocinerea* specimen used in this dissertation, naturally thriving in the polluted brownfield and able to resist high As concentrations *in vitro*, presented a reduction in growth and biomass as compared to those plants grown on a control substrate. This indicates that the presence at high concentration and interference of pollutants other than As reduce the tolerance capacity of *S. atrocinerea* to acclimate and growth in a soil contaminated with multiple TEs.

Bioaugmentation by inoculation of *Pantoea* sp. and *R. erythropolis* in the polluted soil of this study decreased concentrations of TEs (mg metal kg^{-1} plant DW) in roots and leaves of *S. atrocinerea* plants growing in pots. However, an opposite trend was observed in the field trial. In the pots, bacterial inoculation increased the biomass production of *S. atrocinerea*, probably by lowering plant TE uptake. Consequently, the total amount of TEs extracted by the inoculated plants was not significantly different from the non-inoculated ones and in the long term, inoculated plants could be able to extract a higher quantity of TEs

than non-inoculated plants. In the field, inoculation with *Pantoea sp.* led to higher TE concentrations in leaves and higher phytotoxicity which caused a lower biomass and, therefore, less Cu, Zn and Cd were extracted compared to non-inoculated plants. On the contrary, although inoculation with *R. erythropolis* in the field led to lower TE concentrations than with *Pantoea* sp., a higher TE phytoextraction was obtained due to a higher plants biomass production.

In this study, as already mentioned above, the pH could be a major driving force for the differences observed between pot and field but this could be also attributed to differences in root exudation from host plants and/or the concentrations or availability of microbial metabolites and soil nutrients, in an environment subjected to the influence of climatic conditions or inoculant losses due to leaching in the field. Besides, a greater TE accumulation in pot that in field can be because of the restricted volume of soil prospected by the roots, and thus the better ion uptake in pots. Altogether, these results highlight the complexity of soil-microbe-plant interactions and the need for more studies in this direction. Furthermore, despite a contradictory gene expression pattern, condensed tannins were higher again in inoculated plants in both control and polluted soil types, and the same was observed in the field, emphasizing the biocatalyst potential of root-associated bacteria.

The potential application of the bacteria isolated from *S. atrocinerea* can cross the interspecies barrier as seen by inoculation of root-associated *Pantoea* sp. of *S. atrocinerea* to *Arabidopsis thaliana*. By comparing the gene expression of the wild-type (WT) and *cad2-1* mutant (GSH-deficient), in non-inoculated conditions and inoculated with *Pantoea* sp., it was shown that bacterial inoculation increased As accumulation in roots of WT plants. However, a higher presence of As III in the roots of both genotypes caused a different translocation response, based on the genotype sensitivity to As: enhanced growth and accumulation of As in the WT and decreased growth and accumulation in the *cad2-1* mutant. Accompanying this, it was observed that exposure to As results in differential regulation of genes involved in the Pi uptake and starvation response, a downregulation of arsenite transporter *NIP7;1*, the induced expression of genes related to sulfur metabolism (including induction of metallothioneins *MT1a* and *MT2a* transcripts only in the GSH-deficient *cad2-1*), and a differential regulation of oxidative stress-related genes.

The studies carried out in this work, including the differential responses in the different experimental conditions, have shown, in the first place, the crucial need to understand at the molecular and biogeochemical level the mechanisms that underlie the substrate-microorganism-plant interaction; and in the second place, that its application in

phytoremediation, as well as in other biotechnological processes, involves a correct selection of bacteria and plant, suitable to the needs and specific objectives in each case.

IV. CONCLUSIONS

The major contributions of this dissertation are as follow:

1. The interactions between roots, microbes and soils have an effect on the root microbiome and explains the differences in the composition and diversity of the bacterial communities in the soil, rizosphere and endosphere of *S. atrocinerea*.

2. The presence of diverse plant-growth-promoting traits (e.g. production of hormones, siderophores...) in the root-associated bacteria of *S. atrocinerea*, indicates that the relationships at the cellular level between the bacteria and their host are implied in the accumulation and tolerance to pollutants by the plant. This opens new possibilities to assist plants via bioaugmentation under chronic pollutant-stress.

3. Under the presence of arsenate in hydroponic conditions, willow plants show a transcriptional regulation of genes involved in stress responses, arsenate reduction, nutrient transporters, glutathione synthesis and sequestration of As into the vacuoles, which coincides with a rapid arsenite presence and accumulation in root tissues, altered nutrient profile and *de novo* synthesis and increase of non-protein thiolic compounds which contribute to the plant tolerance to the metalloid.

4. Under *in vitro* conditions, it was shown how arsenic uptake by the plant relies on the As concentration available in the culture medium and, more importantly, on its speciation state; and this is affected by bacterial inoculation. Therefore, plant inoculation with *Pantoea* sp. or *R. erythropolis* constitutes a synergistic mechanism that alters arsenic toxicity by changing its speciation, mobility and accumulation in *S. atrocinerea*.

5. In pot, the reduced TE accumulation obtained in inoculated plants with *Pantoea* sp. or *R. erythropolis* in a polluted soil led to a higher biomass in comparison to non-inoculated plants, that in the long-term can increase the phytoextraction of TEs.

6. In field, bacterial inoculation resulted into a higher As and Pb accumulation in the plant and also a greater phytoextraction, together with a markedly higher Zn translocation. While in plants inoculated with *Pantoea* sp. this was achieved by a higher accumulation

of TEs with a biomass decrease, in *R. erythropolis* it was obtained by a lower accumulation but higher biomass than in non-inoculated plants.

7. Given the Zn and Cd accumulation and root-to-shoot translocation rates in willow, together with the As and Pb accumulation in roots and its ability to grow in this area, *S. atrocinerea* is a suitable species to be used in the phytoremediation of soils polluted with these TEs. In addition, bioaugmentation with *Pantoea* sp. or *R. erythropolis* can reduce the time necessary for the soil remediation.

8. Considering that the results obtained in the lab cannot always be extrapolated to the field, the need of performing field assays to obtain successful results in phytoremediation is deduced. However, considerable knowledge on As tolerance, bioaugmentation-based remediation processes and plant-bacteria interaction has been gathered from controlled studies.

9. The selected bacteria have shown a great potential in phytoremediation, not only because of their ability to transform and detoxify pollutants like As, but also for their potential to synthesize enzymes like phytases or arsenate reductases as shown in their bacterial genome. Furthermore, bacteria were able to induce accumulation of certain molecules of interest in plants like tannins or hormones, which emphasizes their biocatalyst potential.

10.   The use of bacteria isolated from *S. atrocinerea* growing in As-polluted soil can also increase As accumulation and tolerance in other plant species, as it is proved in *Arabidopsis thaliana*. Besides, cross-species bacteria inoculation provides more information about the mechanisms implied in bacteria-plant interaction that are needed to tailor specific pollutant-bacteria-plant remediation strategies.

Graphical abstract

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In open-air pots

Chapter 4
In vitro

Chapter 5
In the field

Chapter 6
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root-associated bacteria in
other plant species

Inoculation of *S. atrocinerea*
with the selected bacteria:
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Chapter 3
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for As tolerance and
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Chapter 1

General introduction

1   General introduction

1.1   SOILS AND TRACE ELEMENT POLLUTION

Soils must be understood as the base and support for life and are a non-renewable resource which perform many functions and deliver services vital to human activities and to ecosystem survival (Keesstra et al. 2016). From the biological point of view, soil is defined as the top layer of the Earth's crust, formed by mineral particles, organic matter, water, air and living organisms. Furthermore, soil is the interface between earth, air and water, and hosts most of the biosphere (CEC, 2006). Contamination is the presence of a substance where it is not expected to be or above its background levels (Chapman, 2012) and the term polluted site refers to the one where hazardous substances are present in a level that pose a significant risk to the environment and human health (Payá and Rodríguez, 2018). Soil contamination is a worldwide problem and about 3.6 million sites in the European Union are estimated to be potentially contaminated, with 14% of those sites being highly contaminated and in need of remediation (Payá and Rodríguez, 2018).

Chemical elements in soil are referred to as trace elements (TEs) because of their occurrence at concentrations less than 100 mg kg^{-1}. Trace elements have also been termed 'toxic metals', 'trace metals' or 'heavy metals' (Hooda, 2010). Although, as a matter of fact, many of these elements are present at concentrations much lower than this, whereas other like iron (Fe)

Fig. 1.1. Overview of contaminants affecting soil and groundwater in Europe. TEs: Trace Elements. PAH: Polycyclic Aromatic Hydrocarbons. BTEX: Aromatic Hydrocarbons. CHC: Chlorinated Hydrocarbons (adapted from Panagos et al., 2013)

exceed those limits. Trace elements are the main contaminants affecting soil and groundwater in Europe (**Fig. 1.1**). Most of the TEs of environmental and human/animal health significance are metals [e.g. cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg), zinc (Zn)...]. Other important TEs belong to the metalloid group [e.g. boron (B), arsenic (As)...]. The origin of TEs in soil may be either natural (lithogenic) or anthropogenic (human-induced) (Antoniadis et al. 2017). On a large scale, the lithogenic derivation of TEs, like weathering (including erosion and deposition of wind-blown particles), volcanic eruptions,

forest fires and biogenic sources, are the most important factor affecting total TE content on top soils (Tóth et al., 2016a). Anthropogenic inputs are mainly related to industrial activities, which exploit land for mineral extraction, manufacturing, waste disposal and aerial deposition of fumes; as well as agriculture, such as use of wastes, fertilizers, and human-induced erosion (Kabata-Pendias, 2010; Rinklebe and Shaheen, 2014; Bonet et al., 2016; Mahar et al., 2016). The anthropogenic activities are the major causes of TE accumulation (Shaheen and Rinklebe, 2015), especially in the geographical vicinity of the polluting industrial activities (Li et al., 2015, Gallego et al., 2015). The quantity of TEs that can be retained by a soil or soil constituent is a complex function of surface properties and a variety of environmental and physicochemical parameters (Harter and Naidu, 2001). Therefore, elevated levels of TEs in the soil pose a range of environmental and health risks. Trace elements, unlike organic contaminants, can be retained in soils indefinitely because they are not degradable and constitute a long-term risk of increased plant uptake and leaching (Álvarez-Ayuso et al., 2008), with deleterious implications for the wider environment, including human health. Arsenic, Cd, Hg and Pb are among the most important in terms of the food chain contamination (a phenomenon called biomagnification) and ecotoxicity viewpoints (Tchounwou et al. 2012, Tóth et al. 2016b).

1.2 TRACE ELEMENTS AND PLANTS

The essential elements required by plants in relatively large amounts (>0.1% of dry mass) are macronutrients: carbon (C), hydrogen (H), oxygen (O), nitrogen (N), sulfur (S), phosphorous (P), calcium (Ca), potassium (K) and magnesium (Mg). Additionally, some TEs, such as boron B, Cu, Fe, manganese (Mn), or Zn, are indispensable for the normal plant growth and development, and therefore are regarded as essential micronutrients (Williams and Salt, 2009). The overall inorganic component of cellular and organismal systems is referred to as the ionome (Salt et al., 2008). All of these elements are necessary for the normal functioning of plant metabolism, participating in redox reactions or acting as cofactors of key enzymes or proteins. For instance, B intervenes in organization of cell walls, membrane function, and metabolic activities (Bolaños et al., 2004), Fe-containing proteins play a variety of vital roles in cellular respiration, intermediary metabolism, oxygen transport, and DNA stability and repair, as well as photosynthesis (Li and Lan, 2017), and Zn is required in several plant metabolic processes as an enzyme cofactor (hydrogenase, carbonic anhydrase, RNA polymerase, etc.), maintaining of ribosomal integrity, synthesis of cytochrome and chloroplast development and function (Sharma et al., 2013a). To obtain

sufficient quantities of essential elements, plants have developed flexible and adaptive strategies to avoid excessive accumulation which can result in toxicity, including communication between roots and shoots to match nutrient demand via soil acquisition and to respond appropriately to environmental fluctuations (Taiz et al., 2015). Trace elements such as the metals Cd and Hg, and the metalloid As have no demonstrated biological function in plants, but are nevertheless taken up and cause severe toxicity in most plant species (Morkunas et al. 2018).

1.2.1   *Trace element uptake and transport*

The presence of sufficient quantities of essential nutrients in a soil does not guarantee the availability of these nutrients to growing plants, because other factors such as soil-moisture content, soil temperature, pH, soil physical conditions, presence of toxic elements and/or salts may be limiting their availability (Fageria and Baligar, 2005). Plants only take up from the soil those TEs that are in ionic form and available, that is to say those present as soluble components in the soil solution or solubilized by roots exudates (Chibuike and Obiora, 2014). The availability of these elements depends on their speciation, the binding components, the physicochemical characteristics of soil, the presence of other TEs, the environmental conditions and the biological activity in the soil (Olaniran et al., 2013). Among all these considerations, Harter (1983) reported that soil pH is the major factor affecting metal availability in soil. Available TEs are taken up from the soil by the means of specific transmembrane transporters located in root cells that introduce them into the cytosol (Graham and Stangoulis, 2003, Socha and Guerinot, 2014). However, it does not appear to be transporters designed specifically for the entry of most toxic elements into plants cells, so it has been postulated that some of these TEs gain entry into target cells through the mechanisms of ionic and/or molecular mimicry, by the transporters of essential elements and/or molecules (Bridges and Zalups, 2005).

Trace element accumulation in roots can be due to insolubilization (e.g., at the root surface and in the root apoplast) (Kosegarten and Koyro, 2001) or to a compartmentation in cells avoiding the release to the xylem (Richau et al., 2009). However, a number of metal transporters must exist to mediate metal release to the apoplast and subsequent metal uptake at the pericycle, whereas other metal transporters mediate their release into the xylem (González-Guerrero et al., 2016). This loading involves transmembrane transporters such as those from the families HMA (Heavy Metal-transporting ATPase), MATE (Multidrug And Toxic compounds Extrusion), ABC (ATP-Binding Cassette transporters) or OPT (Oligopeptide Transporter) (Plaza et al., 2007; Arnetoli et al., 2008; Verbruggen et al., 2009;

Verkleij et al., 2009; Maestri et al., 2010; DalCorso et al., 2013). Then, TEs are transported in the transpiration stream with the xylem sap from the roots to transpiring shoot parts (e.g., photosynthesizing leaves) (Page and Feller, 2005) (**Fig. 1.2**).
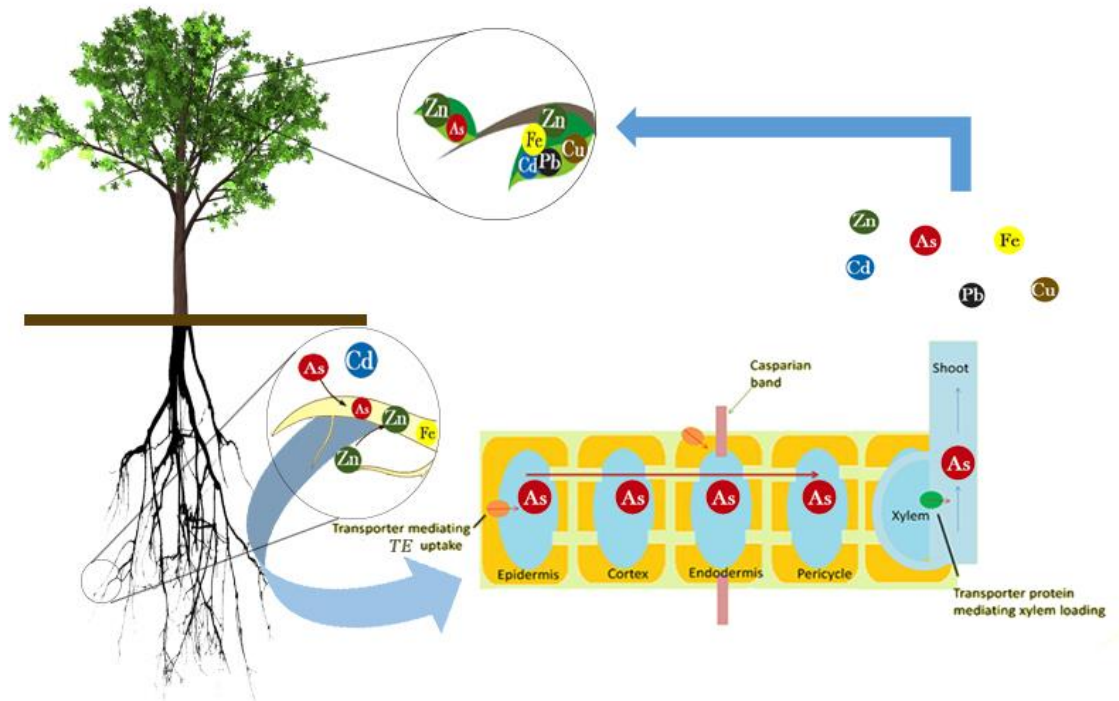
Fig. 1.2. Trace elements (TEs) transportation in the transpiration stream with the xylem sap from the roots to transpiring shoot parts (adapted from Singh et al., 2016).

1.2.2   *Toxicity of trace elements*

After their uptake and transport into the plant tissues, free TEs ions exerts their toxicity through three major mechanisms (**Fig. 1.3**):

•   Due to their chemical similarity with some nutritional elements, TEs compete for the same transporters in root cells, which leads to nutritional deficiencies (Clark et al., 2000). In addition, once inside the cells TEs can displace essential metal ions in metalloproteins, interfering with the biological activity of important enzymes and signaling proteins (Tamás et al., 2014). Examples include the replacement of essential Zn by Cd or As, replacement of K by thallium, replacement of phosphates by arsenate, and mimicry of Mn in place of Fe (Keil et al., 2011).

•   Trace elements bind with high affinity to free thiols or other functional sulfhydryl and carboxyl groups of important proteins, pigments or enzymes. Since these functional

TOXICITY PATHWAYS OF TRACE ELEMENTS

CHEMICAL SIMILARITY

ADP + P

1- arseno-3-phosphoglycerate

1, 3- Diphosphoglycerate

Prevention of formation of ATP

Additional process leading to formation of ATP

No ATP formation

Formation of ATP

ATP

DEFICIENT ENERGY TO METABOLISM

HIGH AFFINITY BINDING TO SULFHYDRYL

FOLDED PROTEIN

Arsenite

Protein

UNFOLDED PROTEIN

FUNCTIONAL INHIBITION OF MOLECULES

GENERATION OF REACTIVE OXYGEN SPECIES

Haber-Weiss reaction

Fenton reaction

Photosynthesis

Redox control

ROS

Plant hormones

Transcription factors

CELL HOMEOSTASIS DISRUPTION

Fig. 1.3. Trace elements major toxicity pathways (adapted from Ayala et al., 2014 and Siddiqui et al., 2019).

groups are often needed for their activity or stability, this can seriously inhibit the functionality of these biomolecules.

• Redox-active TEs, such as Fe, Cu or Mn, can directly enhance the generation of reactive oxygen species (ROS) through Haber-Weiss and Fenton reactions, causing oxidative stress in plant cells (Das and Roychoudhury, 2014). Additionally, non-redox-active TEs such as Zn, As, Cd, Hg or Pb can indirectly lead to the generation of ROS by disrupting the respiratory chain or the antioxidant defense system (Jozefczak *et al.*, 2012; Viehweger, 2014). The excess of ROS can result in cell homeostasis disruption, DNA strand breakage, defragmentation of proteins, or cell membrane and damage to photosynthetic pigments, which may trigger cell death (Emamverdian et al., 2015).

1.2.3 *Trace elements detoxification mechanism in plant cells*

Plants can present different tolerance mechanisms in response to excess of TEs based on adaptation of constitutive mechanisms involved in the general homeostasis of essential TEs ions (Hall, 2002). These mechanisms usually imply maintaining low concentrations of free TEs ions in their cells to prevent the damaging effects described above (Verkleij *et al.*, 2009), and include a reduction in transport through the membrane, exclusion, chelation by glutathione (GSH), phytochelatins (PCs), metallothionein (MT), aminoacids such as histidine, and organic acids and metal compartmentalization in subcellular

structures (e.g. vacuoles), as well as induction of heat shock proteins (HSPs) (Viehweger, 2014; Hasan et al., 2017). For the transport of the TEs-ligand complexes to the vacuoles is again necessary the presence of transporters in the tonoplast membrane, such as the ABCs and the cation/H+ exchanger (CAXs) (Viehweger, 2014). Furthermore, to cope with TEs-induced oxidative stress, plants possess an extensive scavenging system for ROS excess. This include enzymatic (catalase, peroxidases, superoxide dismutase…) and non-enzymatic antioxidants (GSH, some phenolic compounds, proline, carotenoids…) (Mourato *et al.*, 2012; Viehweger, 2014). These antioxidants remove ROS by oxidizing themselves, and so inhibit other oxidation reactions that would damage the cells (Bhaduri and Fulekar, 2012).

The tripeptide (γ-Glu-Cys-Gly) can act as a ROS scavenger via GSH-related enzymes such as GSH reductase (GR), GSH peroxidase (GPX), and GSH sulfotransferases (GSTs), as an electron donor in redox reactions, as metal chelator and as substrate for the synthesis of PCs (Asgher et al. 2017). Therefore, this low-molecular-weight tripeptide is the principal low molecular weight thiol in plants (Schnaubelt et al., 2013). The diverse functions of this key biomolecule originated from the sulfhydryl group in its cysteine residue (-SH), which due to its exceptional electron-donating capacity allows GSH to chelate metals and participate in redox cycling (Meyer and Hell, 2005; Jozefczak et al., 2012).

1.3   SOIL REMEDIATION

Remediation of contaminated soils refers to reducing the harmful environmental and human effects of the exposure to hazardous substances. Besides trying to reduce TEs exposure, the present and future use of the remediated site is also taken into account (Burger and Gochfeld, 2016). Assessing the availability and speciation of TEs is crucial for determining the environmental impact of contaminated soils, as the total TE content of soils alone is not a good indicator/predictor of their plant uptake or ecotoxicity (Hooda, 2010). Numerous availability and speciation assessment soil tests have been used and proposed (McLaughlin et al., 2000), but so far there is no general consensus on how metal speciation and availability can assist the risk-assessment process.
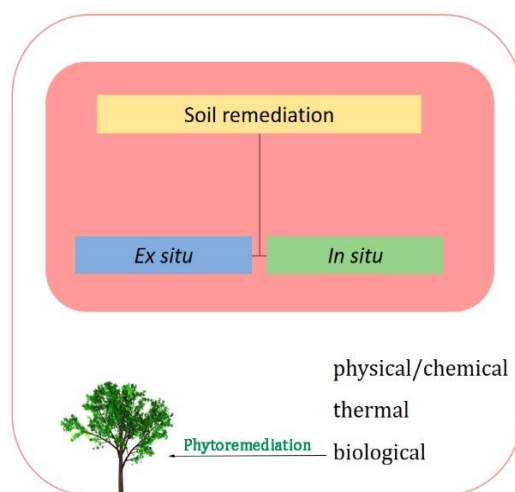

Fig. 1.4. Soil remediation strategies.

Taking into account the number of contaminated sites in the European Union and the area affected by different kinds of pollution, the European Commission has estimated that managing contaminated land costs around € 6.5 billion per year (Payá and Rodríguez, 2018). The regulatory limits for maximum permissible concentrations of TEs in soils differ between European Union Member States. Therefore, the definition of what clean means will depend on national (or even regional in the case of Spain) regulations. To achieve this, there are many techniques available for soil cleaning. The two main types of remediation are *ex situ* and *in situ* (Kidd et al., 2015) (**Fig. 1.4**). *Ex situ* involves physically extracting media from a contaminated soil and moving it to another location for treatment. At an *ex situ* site, if the pollutant exists only in soil, the soil is excavated. If contamination has reached the groundwater, it is then pumped and both the polluted soil and water are removed. Despite soil excavation, disposal and containment to landfill is a fast method that provides undisputable and easily determined results, it comes with a high costs due physical excavation, transportation of polluted soil out to a landfill and transportation of replacement fill back to the site. Dumping polluted material at a landfill is subject to high and rising admission fees and taxes. Consequently, remediating sites with large contaminated volumes using off-site ex-situ or mass-transfer impose a high cost (Tangahu et al., 2011; Ali et al., 2013). On the contrary, *in situ* remediation involves treating contaminants on-site (Kuppusamy et al., 2016). There are multiple techniques for *ex situ* or *in situ* treatments that can be categorized in three main groups (United States environmental protection agency (US EPA), 1996) (**Fig. 1.4**): physical/chemical, thermal and biological technologies.

1.3.1 Phytoremediation

Phytoremediation, already described more than two decades ago (Raskin et al. 1994), is a cost-effective and environment-friendly cleanup green technology that exploit the ability of certain plants to accumulate TEs in their tissues, thus reducing soil contamination or rendering these elements harmless (Pilon-Smits, 2005; Mahar *et al.*, 2016). This phytotechnology is especially appropriate when other remediation methods are not practicable (Ali *et al.*, 2013; Chibuike and Obiora, 2014). Nevertheless, phytoremediation has also limitations, being the most recognizable that it is usually slower than other conventional remediation techniques (Khan *et al.*, 2000; Pilon-Smits, 2005). Besides, plants have to be in contact with the pollutants and be able to grow in the polluted soil; therefore, phytoremediation is restricted by the capacity of roots to explore the soil and the tolerance

of plants to TEs. Additionally, the limited available fraction of pollutants in the soil makes this phytotechnology less suitable for full decontamination (Vangronsveld *et al.*, 2009). It has been stated that the clean-up time should not exceed 10–15 years for an economically feasible soil remediation (Vangronsveld *et al.*, 2009; Mench *et al.*, 2010); therefore, to allow phytoremediation within a reasonable period of time, it is necessary to drastically improve the metal uptake and/or biomass production (Khan et al., 2000). The use of soil amendments to enhance pollutants availability can potentially optimize the efficiency of phytoremediation, although this is controversial as, at least in the short-term, it increases the risk of spreading the contaminants into the environment (Bolan *et al.*, 2014).

Phytoremediation includes the following strategies (Pilon-Smits, 2005; Favas et al., 2014; Sarwar et al., 2017) (**Fig. 1.5**):

- **Rhizofiltration**: absorption of contaminants from polluted waters.

- **Phytostabilization**: stabilization of pollutants in the soil by precipitation, sorption, metal valence reduction, or complexation. preventing the erosion, leaching or runoff.

- **Phytoextraction**: uptake of pollutants by the plants and accumulation in their harvestable parts.

- **Phytodegradation**: degradation of organic pollutants by plant enzymes.

- **Phytovolatilization**: uptake of pollutants from the soil, transformation into volatile forms and subsequently release into the atmosphere.

- **Phytostimulation**: plant-assisted degradation of organic pollutants in the rhizosphere due to microbial activity.
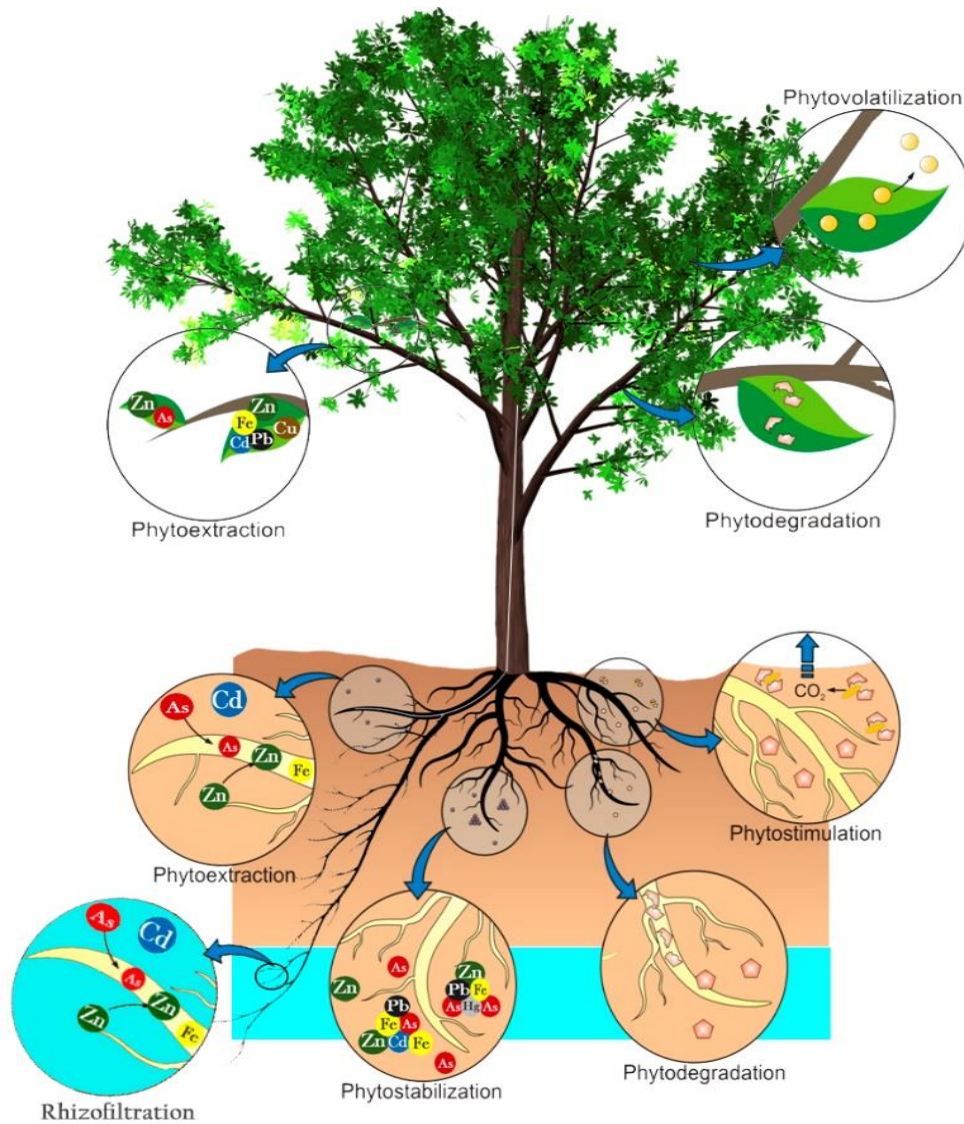
Fig. 1.5. Schematic representation of phytoremediation strategies (adapted from Favas *et al.*, 2014).

The debate over the efficacy of delivering suitable microorganisms to enhance phytoremediation has gained increased popularity in the last 30 years. This **bioaugmentation** process can promote plant health and growth, enhance water and mineral uptake, and even inhibit the growth and development of pathogenic soil microorganisms (Pilon-Smits, 2005). Many microorganisms are capable of degrading toxic organic compounds and microbial processes play a major role in TE cycling in the plant-soil-microbe system (Lampis et al., 2015). Therefore, an integration of microbial bioremediation and phytoremediation is a more promising strategy for remediation of contaminants (Kong and Glick, 2017a).

1.3.2 *Plant species selection in phytoremediation*

One of the most complex problems faced when optimizing a phytoremediation program is certainly to select the plants that should be used. TEs-induced stress cause, among other deleterious effects, loss of plant biomass associated with growth inhibition (Gill et al., 2015). Nevertheless, a number of plant species and soil biota populations, usually endemic of polluted soils, are able to colonize and thrive in such environments, even when high concentrations of TEs are found in their cells or tissues (Thijs et al., 2017). However, although plants growing in these areas are tolerant to the predominant natural occurring TEs, in most of the cases, they show slow growth and have a limited biomass (Maywald and Weigel, 1997). This is a fundamental impediment to use hyperaccumulator plants in phytoremediation programs, because fast growing and deep-rooting traits are required for effective phytoremediation of most polluted soils (Fernández et al., 2010). Related to this, studies exploring the feasibility of high biomass plants to extract metals from polluted soils, such as willow (*Salix viminalis*), have demonstrated that the high biomass compensates for the moderate TE concentrations found in the aboveground tissues of such species (Hammer et al., 2003).

Then, a good practice to implement when selecting plants to phytoremediate highly polluted soils, is the selection and characterization of native hyperaccumulator plants with the highest biomass possible. These plants growing spontaneously in those polluted soils, are better adapted to the particular climatic and soil conditions of the site. After measuring their TE content, species selection should consider their ability to accumulate TEs, amount of biomass, percentage cover/aggregation and frequency of appearance in polluted areas. It is then important to identify the characteristics that make the chosen plants more prone to hyperaccumulate TEs, such as being nitrophilous, ruderal or resistant to other types of abiotic or biotic stresses (Fernández et al., 2010). Later, the most promising TE-accumulator plant propagated from seeds can be selected and cloned *in vitro* to be used in phytoremediation programs (Fernandez et al., 2008, Fernández et al., 2012) (**Fig. 1.6**). Besides, the responses of plants and soil organisms to contamination are mutually dependent (Krumins et al., 2015) and, as far as microorganisms are concerned, TEs-contamination decreases microbial abundance, diversity and activity (Chen et al., 2014). It is well-known that the plant-associated microbial community plays an important role in adapting plants to extreme environments (Yuan et al., 2016). Therefore, studying the isolated indigenous bacteria and their interaction with the plant may provide a better understanding of TE mobility and tolerance in the phytoremediation of contaminated fields (Mesa et al., 2017) (**Fig. 1.6**). As a consequence, in many of the phytoremediation examples
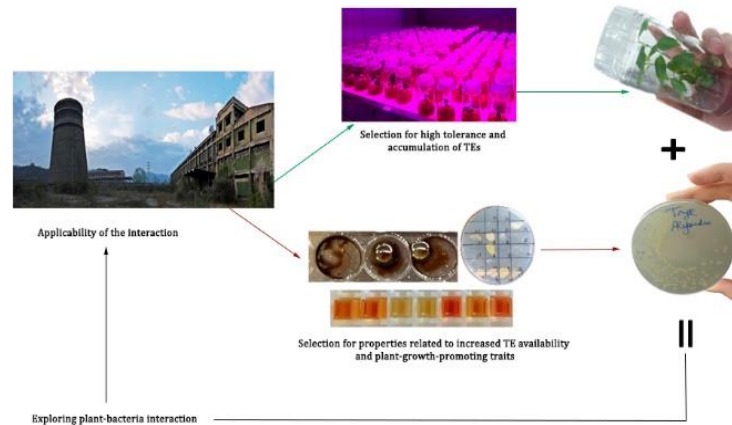
reported in the literature a comprehensive study would be still required to explore the genetic basis for TE tolerance and the microflora interaction effects on accumulation (Kong and Glick, 2017b).

Despite most authors consider that native plant species are the best option for a phytoremediation process, plants adapted to pedo-climatic and environmental conditions similar to those reigning in the polluted site may also constitute a suitable and affordable possibility (Kazakou et al., 2008). Nonetheless, introduction of non-native hyperaccumulating

Fig. 1.6. Schematic representation to improve phytoremediation of highly polluted soils with trace elements (TEs), based on selection and characterization of bacteria and plant properties and their interaction.

species may incur in side-effect environmental problems and increase the costs of the phytoremediation process. Such strategy may need additional environmental monitoring to prevent unforeseen spreading of contaminants or competition with natural flora and microbiota, and may require prevention of unintentional cross-fertilization and hybridization with local plants, which may reduce phytoremediation effectiveness (Che-Castaldo and Inouye, 2015).

1.3.3   *Salix atrocinerea*

As we have pointed already, the high biomass production and massive deep root systems of woody plants have caused an increasing interest in the use of fast growing tree species for phytoremediation in the last decades which could make this phyto-technology economically viable (Rosselli et al., 2003, Rockwood et al., 2004, Unterbrunner et al., 2007). *Salix* is a diverse genus with about 450 species (Lauron-Moreau et al., 2015), that generally take up a lower concentration of trace elements per gram of tissue but, due to their higher biomass production, can extract comparable net amounts of pollutant (Purdy and Smart, 2008). *S. atrocinerea*, commonly called grey willow (**Fig. 1.7**), has a Mediterranean and South-European Atlantic distribution type (Skvortsov, 1999). Therefore, as a native species present in the area to decontaminate, the use of *S. atrocinerea* in phytoremediation has an

Fig. 1.7. Detail of *Salix atrocinerea* leaves growing spontaneously in the field of study.

1.4 BACTERIA-ASSISTED PHYTOREMEDIATION

As previously mentioned, the success of phytoextraction depends on TE availability to the roots and the ability of the plant to intercept, take up, and accumulate trace elements in shoots. On the other hand, the fitness/performance of plants is determined by the entire suite of genes (the so called hologenome), consisting of the genome of the host plant plus the genomes of all their associated microorganisms (Mitter et al., 2016). Thus, when aiming to improve plant nutrition or pollutant extraction in a sustainable manner, the role of plant-associated microorganisms should also be regarded (East, 2013; Mitter et al., 2016). Within a plant, different compartments can be identified, each with a different microbial community, such as the rhizosphere (the portion of soil directly affected by TEs and root exudates) or the endosphere (the internal tissues of the plant roots) (**Fig. 1.8 A, B**). Since Hiltner's pioneer work dating back from last century (Hiltner, 1904), the composition and role of the plant microbiome has been studied. Bacteria typically found in association with TEs-tolerating or accumulating plants were initially identified by culture techniques, and their potential involvement in plant growth, TEs accumulation in plant tissues and efficiency and rate of phytoextraction, analyzed (Sessitsch et al., 2013). However, most microbes are not cultivable in the laboratory, because we do not know their specific nutritional and environmental requirements and therefore, their ecological role (Pande and Kost, 2017). Alternative methods to study them are based on the sequencing of their DNA, after directly isolating the genetic material of living or dead cells from environmental samples. Metagenomics is the molecular tool that helps to understand this genetic composition and therefore to explore the diversity of microbial species within the wide variety of

uncultivable microorganisms. Currently, one of the most advanced technologies used in metagenomics studies is next-generation sequencing (NGS), that, together with the development of different computational tools for the analysis of large sets of metagenomic data, has revolutionized the field of microbial ecology. These techniques allow the massive sequencing in parallel of multiple samples at a considerably low cost compared to the previous methods (De Mandal et al., 2015). Since the advent of NGS, there has been a shift away from targeted isolation of small numbers of microbes towards large-scale projects aimed at sequencing the entire microbial population within an environmental niche (Farrar et al., 2014) and to use this knowledge to improve phytoremediation (Thijs et al., 2016).
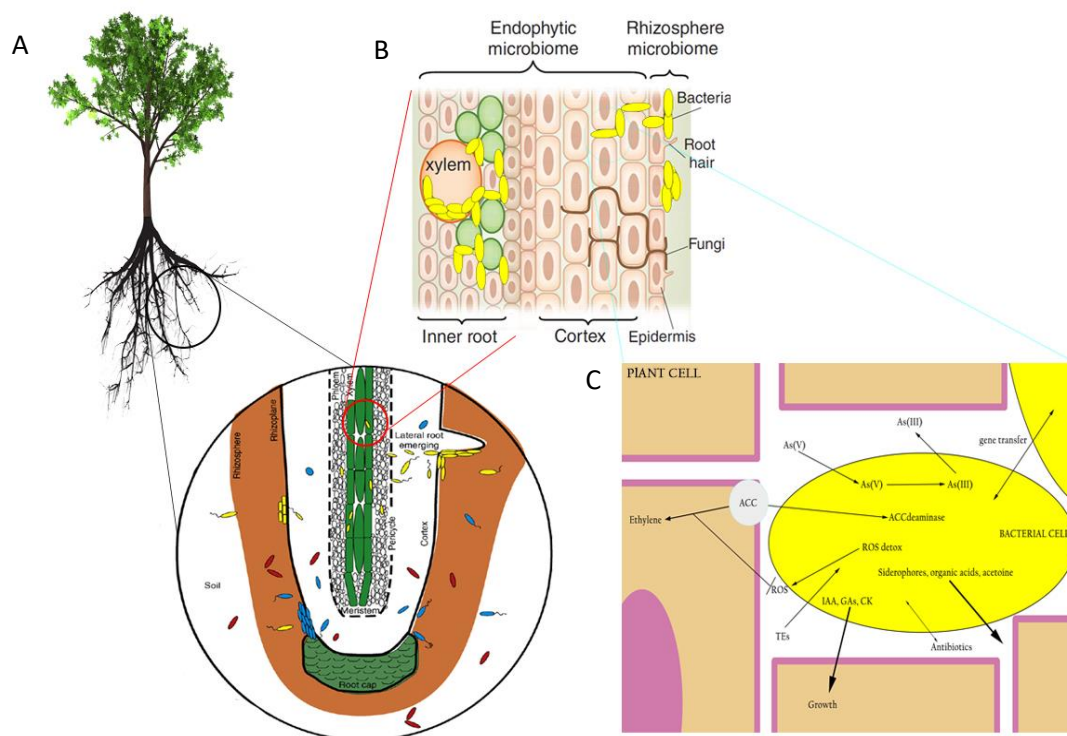
Fig. 1.8. Types of root-associated bacteria and their root colonization process (Hardoim et al., 2008) (**A**) and (Hirsch and Mauchline, 2012) (**B**). Bacteria with the capacity to synthesize plant hormones, such as indole-3-acetic acid (IAA), gibberellins (GAs) and cytokinins (CKs) can affect plant growth and development, as well as trace element accumulation via de production of enzymes (e.g. arsenate reductase, ACC deaminase) and other bacteria metabolites (e.g. siderophores, organic acids, acetoine) (inspired by Hardoim et al., 2015) (**C**).

However, only cultivable bacteria can be isolated and fully characterized in the laboratory to evaluate they further use in bioaugmentation processes. For example, *in situ* bioaugmentation by poplar isolated *Pseudomonas putida* W619 decreased trichloroethylene (TCE) evapotranspiration up to 90% under field conditions (Weyens et al., 2009a). This result was achieved after the establishment and enrichment of *P. putida* W619-TCE as a poplar root endophyte followed by further horizontal gene transfer of TCE metabolic activity to members of the poplar's endogenous endophytic community (Weyens et al., 2009b).

1.4.1   *Bacterial microbiome in the rhizosphere environment*

The rhizosphere is a hot spot for numerous organisms and is considered as one of the most complex ecosystems on Earth (Hinsinger et al., 2009). Organisms found in the rhizosphere include bacteria, archaea, viruses, fungi, oomycetes, protozoa, algae, nematodes and arthropods, which had led to the emergence of the term 'rhizosphere zoo' (Buée et al., 2009). Specifically, the rhizosphere microbiome is of central importance not only for plant nutrition and health, but also contributes substantially to microorganism-driven carbon sequestration, which has an important role in ecosystem functioning and nutrient cycling in terrestrial ecosystems (Berg et al., 2014). In contrast to non-rooted bulk soil, the rhizosphere is characterized by much higher bacterial abundances and activities, collectively termed as "the rhizosphere effect" (Bais et al., 2006). The major driving force in the regulation of the microbial diversity and activity in the rhizosphere soil and rhizoplane (external root surface), and ultimately in the formation of distinctive rhizosphere microbiota from soil biomes, is the deposition of large amounts of organic carbon by the plant roots in a process termed rhizodeposition (Dennis et al., 2010). The composition of the plant root exudates is highly variable between plant species, cultivars and moreover with plant age and developmental stage, resulting in specific bacterial communities (Chaparro et al., 2013). Furthermore, it now appears that in addition to carbohydrates and even amino acids, which act as general chemical determinants in the rhizosphere, secondary metabolites such as plant-specific flavonoids also play a role in the development of these plant-specific microbial communities (Badri et al., 2013, Weston and Mathesius, 2013).

1.4.2   *Bacterial microbiome in the endosphere environment*

In addition to bulk soil and rhizosphere communities, large and diverse bacterial populations, collectively termed endophytes, live inside plants without causing detrimental effects or cellular damage to the plant (Hardoim et al., 2015). For a long time, endophytes were ignored and/or considered as contaminants, but many endophytic inhabitants of plants are now often recognized as having unique, intimate and crucial interactions with the plant (Weyens et al., 2009c, Hirsch and Mauchline, 2012) (**Fig. 1.8C**). Endophytic bacteria reside for at least part of their lives within plant tissues and can be considered to position at the benign end of the spectrum between mutualists and pathogens (Compant et al., 2010). However, they also include latent pathogens, which depending on environmental conditions and/or host genotype can cause diseases (Sessitsch et al., 2012). The majority of endophytes are widely considered as being a sub-population of the rhizosphere microbiome, since their

primary colonization route are the plant roots (Hardoim et al., 2008). However, endophytes also display characteristics distinct from rhizospheric bacteria, suggesting that not all rhizospheric bacteria can enter plants and/or that after colonization of their host plant, endophytes have the potential to modulate their metabolism and become adapted to their internal environment (Monteiro et al., 2011).

In this case, understanding factors that control microbial communities, as well as their function in plant growth, TE availability and tolerance, is essential for designing and supporting microbiome-based phytoremediation.

1.5 THE PROBLEM OF ARSENIC AND TRACE ELEMENTS MULTIPOLLUTED SOILS IN ASTURIAS (SPAIN)

As already mentioned, while natural soil mineral backgrounds might be the reason for high TE concentrations on the affected soils (Herath et al., 2016), historical and recent industrial and mining areas also show elevated concentrations (predominantly of chalcophile elements as As, Cd, Pb and Hg) (**Fig. 1.9A**), indicating the magnitude of anthropogenic effect on soil quality in Europe (Tóth et al., 2016b), and over 200 years of industrialization have caused soil contamination to be a widespread problem, especially in the northern part of Spain (**Fig. 1.9B**). Langreo, a municipality and town of Northern Spain (Asturias) has been one of the most important mining and metallurgical points of Spain (Martínez et al., 2014) (**Fig. 1.9C**). In recent decades the closure of these heavy industry, and the mining crisis have generated many brownfields (defined by the US Environmental Protection Agency, EPA, as property, the expansion, redevelopment, or reuse of which may be complicated by the presence or potential presence of a hazardous substance, pollutant, or contaminant), in the region. The pollution of these brownfields affects the soil and groundwater and constitutes a risk not only for the environment but also for human health, as well as economic and social costs (Gallego et al., 2016). The recovery of these affected areas, especially when they have multi-component contamination and constitute a focus of pollution for the urban or peri-urban zones, is of great interest to governmental authorities (Sierra et al., 2010). Besides, the magnitude of TE dispersal into the environment increases with increasing contaminated soil area, where cleanup strategies become more complex (Morio et al., 2013). The large extension of these areas makes them a perfect example of study, since soils and subsoils may contain complex mixtures of TEs, which appear frequently as multi-contaminant situations. In addition, specific weathering

conditions and different edaphic factors modify chemical speciation and toxicity of these contaminants (Adriano, 2001, Wenzel et al., 2003).

1.5.1   *The case of Nitrastur*

Nitrastur (Langreo, 43°18'49"N, 5°42'05"W, Asturias), one of the main fertilizer factories in Spain for more than fifty years until its closure in 1997 (Gallego et al., 2016), is among the polluted brownfields identified in the Langreo region on a previous study made by the Regional Ministry of the Environment of the Principado de Asturias (CMPA, 2001). The facilities of the factory comprise an area of 20 ha (**Fig. 1.9D**), in which a part has been delimited to perform phytoremediaiton trials (**Fig. 1.9E**). In this area different byproducts and wastes from that and other factories of the surroundings were used as fillers in order
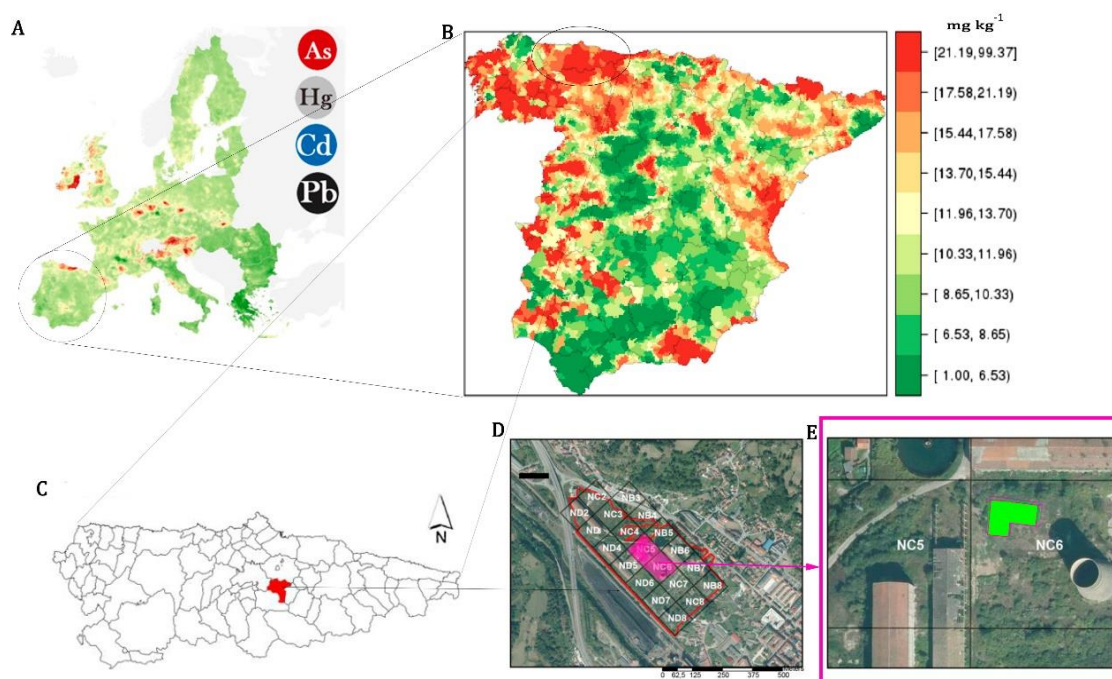
Fig. 1.9. Co-occurrence of chalcophile elements (As, Hg, Cd, Pb) in Europe based on the spatial representation of results of the principal component analysis on different trace elements (Tóth et al., 2016b) (**A**). Municipal distribution of arsenic topsoil concentrations in Spain's mainland (Núñez et al., 2015) (**B**). Location of the fertilizer industry "Nitrastur" site in Spain (Langreo, Asturias) (**C**), aerial view of the site where the red thread delimits the Nitrastur industrial area and the black lines show the sampled grid delineating 100 X 100 m plots (**D**) and zoom of the NC5 and NC6 sampling plots, the phytoremediation area located in NC6 is highlighted in green (**E**) (Mesa et al., 2017).

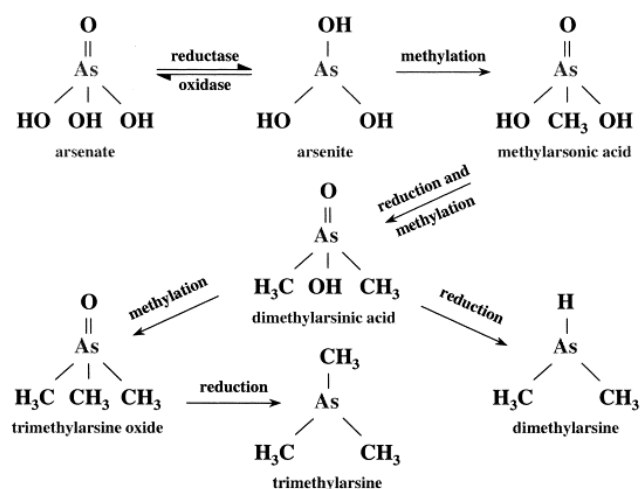to obtain a raised and flat surface. In consequence, the natural soil is contaminated as a result of decades of industrial activity and waste dumping. Among these wastes, the pyrite ashes derived from the roasting of sulphurous minerals for the production of sulfuric acid, are considered the most problematic waste, due to their high levels of As and Pb in combination with the oxides and hydroxides (Gallego et al., 2016).

1.6   ARSENIC IN THE ENVIRONMENT

Arsenic is a widely spread toxic element in the earth's crust and industrial activities have increased its presence over safe limits, since ingestion of As by humans causes health disorders (Monachese et al., 2012). Being the 20th most common element, background concentrations in soil are often around 6.83 mg kg^{-1}, but can range from as low as 0.1 mg kg^{-1} (falling in the category of TE) to as high as 1000 mg kg^{-1} (WHO, 2003). The EPA and the International Agency for Research on Cancer (IARC) have ranked As and its compounds as a Group 1 human carcinogen (Abbas et al., 2018). In this context of potential effects should be interpreted the evidence of increased risk of developing certain types of cancer in areas close to mines that originate arsenic as a by-product (Fernandez-Navarro et al., 2012). Chronic exposure arising from low levels of As in topsoil could be a potential risk factor for developing cancer of the stomach, pancreas, lung and brain and non-Hodgkin's lymphomas (NHL) (Núñez et al., 2016). Ingestion of As also induces changes in intestinal microbiota with still unknown consequences (Gokulan et al., 2018). Therefore, one of the biggest challenge to overcome is efflux of As into drinking water and food (Chain, 2009). The fact that As natural attenuation (the natural soil environment processes which effectively reduce contaminant mobility, bioavailability, toxicity, or concentration to levels that are not overly harmful to human health and ecosystems) is too low (1000-3000 years) (Bowen, 1979), highlights the need to accelerate this process.

1.6.1   Arsenic forms

Arsenic has chemical and physical properties intermediate between a metal and a non-metal, and is often referred to as a metalloid or semi-metal and can exist in four oxidation states: –3, 0, +3, and +5 and its solubility depends on the pH and ionic environment (Panda et al., 2010). Arsenite, As III, and arsenate, As V, are the predominant oxidation states in the environment under, respectively, reducing and oxidizing conditions (Khalid et al., 2017). From a biological and toxicological perspective, there are three major groups of arsenic


Fig. 1.10. Arsenic species in the environment and their transformations (Mukhopadhyay *et al.,* 2002).

compounds (Straif et al., 2009) (**Fig. 1.10**): inorganic arsenic compounds, organic arsenic compounds and arsine gas.

So far there is contradictory evidence to state whether organic forms of As are more lethal compared to inorganic As (Finnegan and Chen, 2012; Duncan et al., 2017). However, it is widely accepted that amongst its inorganic forms, As III is more soluble and up to 60 times more toxic and mobile than As V, as the former has been shown to react with sulfhydryl (–SH) groups of proteins and enzymes, inhibiting the cellular function and eventually causing cell death (Pandey et al., 2017, Abbas et al., 2018).

### 1.6.2   *Arsenic natural resistance and tolerance mechanisms*

As a consequence of the ubiquity of arsenic, nearly every organism has intrinsic or acquired similar mechanisms for As detoxification (Ali et al., 2009, Garbinski et al., 2019) (**Fig. 1.11A, B, C**). These include biotransformations such as oxidation and reduction (Silver
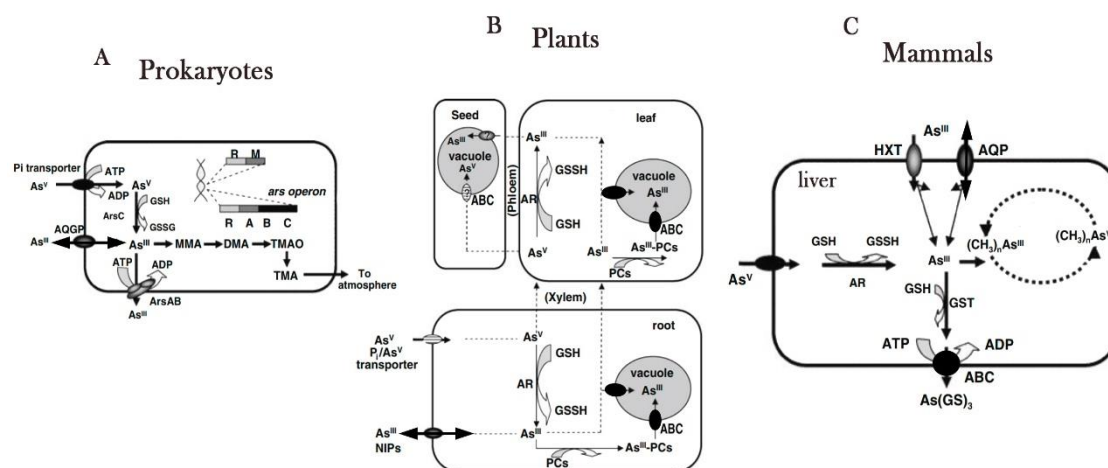
Fig. 1.11. Arsenic metabolism in prokaryotes, plants and mammals. In bacteria the main uptake of arsenate (As V) into cells occurs via phosphate (Pi) transporters. Arsenite (As III) enters the bacterial cells via the aquaglyceroporin (AQGP). As V is reduced to As III by the bacterial ArsC arsenate reductase using glutathione (GSH) as reductant. ArsB or ArsAB ATPase, extrude As III into the external environment. In addition, As III can be released into the environment in volatile form after subsequent methylation steps carried out by As III-S-adenosylmethionine methyltransferase. MMA monomethylarsonic acid, DMA dimethylarsinic acid, TMAO trimethylarsine oxide, TMA trimethylarsine (**A**). Plants take up AsV through phosphate transporters (Pi). As III influx occurs through aquaglyceroporins of the NIP (nodulin like intrinsic protein) subfamily. As V is reduced to As III by arsenate reductase (AR) using glutathione (GSH) as a reductant and As III can form complexes with thiol groups from glutathione and phytochelatins (PCs) to lower its cytotoxicity. The complexed As III and inorganic As III are believed to be mostly sequestered into the central vacuole via as yet unknown transporters. Inorganic As V and As III are the major arsenicals found in the xylem sap of plants. Most plant species act as 'excluders' i.e. a very small proportion of arsenic is translocated to shoot tissue where similar reduction and sequestration mechanisms are present. Via the phloem, some of the total arsenic content ends up in the vacuoles and other tissues of edible parts such as seeds (**B**). In mammalian cells, uptake of As III can occur via aquaporins and via HXTs. Specific proteins responsible for arsenate uptake and arsenate reduction in mammals have yet to be identified. The main efflux mechanisms for As III in mammals appear to be ABC transporters. Methylation of As III by arsenic methyltransferases such as AS3MT increases mobility of arsenicals in the body and facilitates removal through skin and urine (**C**). Adapted from Ali et al. (2009).

and Phung 2005; Abbas et al., 2018). Arsenic uptake by plant species relies on its total concentration and, importantly, on the speciation of As in soil—which is thought to be dependent upon exchangeable (available) As concentration in soil (Martínez-Sánchez et al., 2011) (**Fig. 1.12A**) and the action of microorganisms (Drewniak and Sklodowska, 2013) (**Fig. 1.12B**). In plants, As mainly enters adventitiously as an inorganic form and through same transporter as chemical-related molecules. As III entering in the roots via various nodulin-26-like intrinsic proteins (NIPs) and As V, being analog to phosphate (Pi), enters in the root cells using the same transporters as Pi to cross the plasma membrane of the root cell (Zhao et al., 2009) (**Fig. 1.12C**).

Arsenic accumulation in plant, as in any other biological organism, can affect growth and productivity due to the toxicity mechanisms generally described for TEs in section (**1.2.2**). It has been reported that As V toxicity to plants mainly occur via the replacement of Pi in key biochemical processes (Finnegan and Chen, 2012). Once inside plant cell, As V is reduced to As III with the help of As V reductase, CDC25-like tyrosine phosphatase (Bleeker et al., 2006). The reaction is coupled with NAD(P)H oxidation via GSSH reduction catalyzed by glutathione reductase (GR) (Sharma et al., 2012). This idea is, at first, counterintuitive, since As III is more toxic than As V, but the detoxification of As III is accomplished by the formation of its complexes with thiol-rich peptides, followed by its storage in root vacuoles
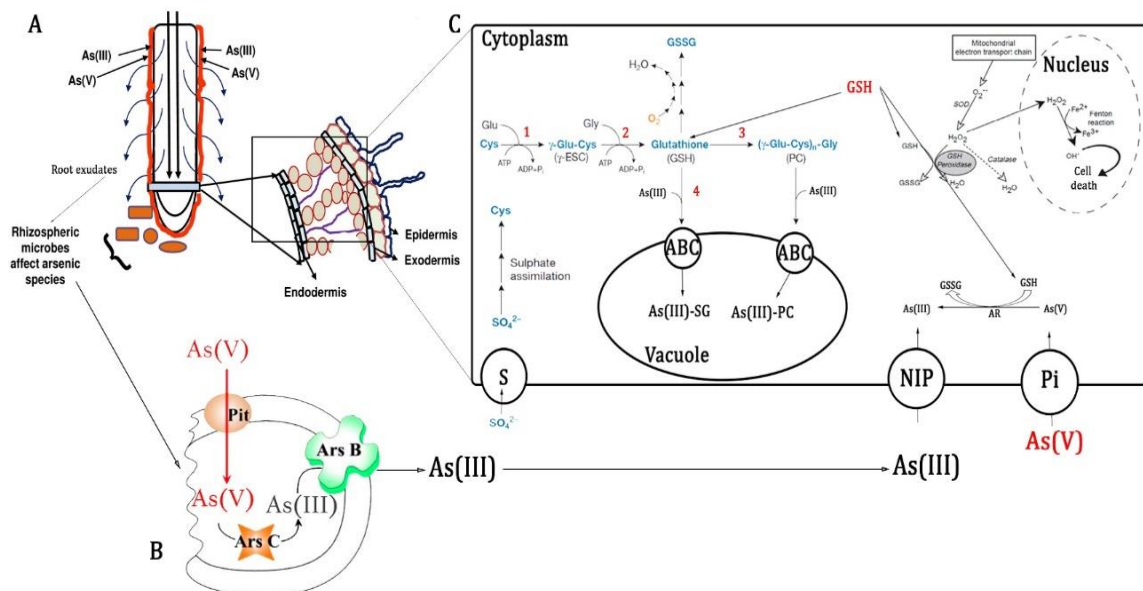
Fig. 1.12. Arsenic (As) uptake by roots is determined by As species availability to plants rather than by total concentration in the soil (adapted from Awasthi et al., 2017) (**A**). Where plant exudates determine the rhizospheric microbes which also play a role in inter-conversion of arsenic species increasing As III presence under As V exposure (adapted from Silver and Phung, 2005) (**B**). Mechanism of detoxification of As and oxidative stress in plant cells by glutathione. Cys, cysteine; γ-Glu-Cys, γ-L glutamyl-L-cysteine; γ-ECS, γ-glutamylcysteine synthetase; GSH, glutathione; GSSG, oxidized glutathione; PC, phytochelatine; As (III)-PC, arsenite–phytochelatine complex; As(III)–SG, As(III)–GSH conjugate. (1) γ-Glutamylcysteine synthetase; (2) glutathione synthetase; (3) phytochelatine synthase; (4) glutathione S-transferase (GST) (adapted from Kurz et al., 2004; and Peuke and Rennenberg, 2005) (**C**).

using the ATP-Binding Cassette (ABC) transporters (Liu et al., 2010, Song et al., 2010) (**Fig. 1.12C)**. This is responsible for very low efflux of As III and its long-distance transport to other tissues of the plants (Liu et al., 2010). If As III was free in the cytosol, it could disrupt proteins with a Zn finger motifs in their structure (e.g., numerous enzymes involved in transcription and DNA repairing). Different studies have described that As III binds to proteins which have three or four cysteine residues in the Zn-binding site, via oxidation of −SH groups, Zn is then replaced by As III and this causes enzyme inactivation (Kroncke and Klotz, 2009). In addition, this As III-replaced molecules are very sensitive to oxidation by ROS and after this As III is further released causing protein damage (Zhou et al., 2015).

Despite As being a non-redox-active TE, it has been described that it increases ROS accumulation in plants through the disruption of electron transport chains (ETC) in mitochondria and chloroplasts, glycolate oxidase activation and antioxidant inactivation (Fayiga and Saha, 2016). It has also been reported that As III, thorugh interaction with the mitochondrial outer membrane proteins, can break them and, consequently, release ROS (Hosseini et al., 2013). The increase in ROS, like hydrogen peroxide (H_2O_2), is quite dangerous for plant metabolism and can cause irreparable damage to important macromolecules (Talukdar, 2017) (**Fig. 12C**). However, it is also worth mentioning, that, despite H_2O_2 is considered a toxic compound, it also plays an important role as signal molecule which participates in the complex network regulating cell responses to As, like ethylene production (Thao et al., 2015, Cuypers et al., 2016). As III binds to a large number of free GSH molecules which are then secreted into the vacuole or out from the cell. Although this process leads to a decrease in As III concentration in the cell, it also decreases GSH pool, thereby reducing the activity of many enzymes using it as a substrate (e.g., of glutaredoxins, which are electron carriers in GSH-dependent syntheses) and decrease the defense against oxidative stress (**Fig. 1.12C**) (Flora et al., 2007).

1.6.3   *Role of microorganisms in arsenic phytoremediation*

The speciation of As is important, As V and As III differ greatly with respect to their phytoavailability, being As V less bioavailable than As III because is more strongly retained by soil constituents (Abbas et al., 2018). Microorganisms can interconvert As III and As V and thus are capable of either solubilizing or immobilizing As in the soil-plant system (Bakker et al., 2013). Different studies have isolated and reported various species of strict aerobic As III-oxidizing and facultative anaerobic As V-reducing bacteria from As-contaminated sites (Coats and Rumpho, 2014). Soil microorganisms increase As bioavailability by releasing/converting As into its more mobile or water-soluble form, As III

(Hu et al., 2015). Some studies also reported an increase or decrease in phytoavalability of As in soil after the inoculation of microbes in soil (Bakker et al., 2013, Abbas et al., 2018).

Nealson et al., (2002) reviewed how for some bateria breathing metals rather than oxygen was a way of life and introduced the term "eating and breathing". For arsenic, "eating" means As III functioning as an electron donor at the start of a membrane respiratory chain (**Fig. 1.13A**), and "breathing" means As V functioning as a terminal electron acceptor for an anaerobic respiratory chain (**Fig. 1.13B**), as do other TEs, such as Fe and Mn with some microbes. However, the most universal and well-characterized As transformation is not coupled to respiration and it is considered as an As-resistance mechanism, and as seen in plants, it is induced by the ars system (Oremland and Stolz, 2003). The arsC cytoplasmic arsenate reductase (**Fig. 1.13C**) is found widely in microbes, and the arsC gene occurs in ars operons in most bacteria as well as in some archaeal genomes (Silver and Phung, 2005).
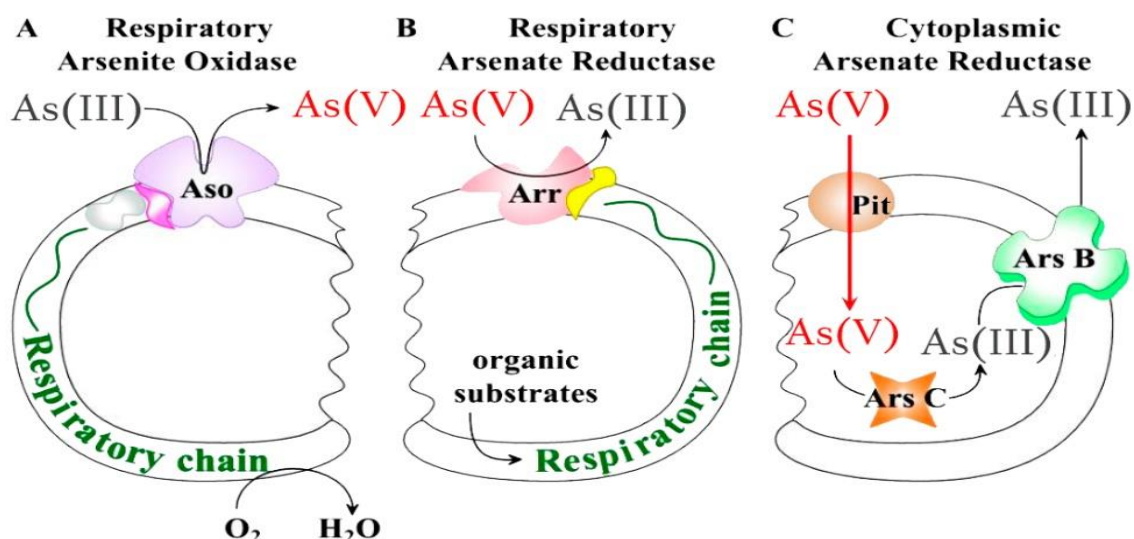
Fig. 1.13. Cellular locations and functions of bacterial respiratory arsenite oxidase (**A**), respiratory arsenate reductase (**B**), and cytoplasmic arsenate reductase (**C**) (adapted from Silver and Phung, 2005)

Despite the whole knowledge about potential transformations of As by bacteria, relatively less information is available on accumulation, mobilization, distribution, and speciation of As by root-associated bacteria and their impact on plant growth and development (Remans et al., 2012; Pandey et al., 2018). So far, under As exposure it has been observed that colonization of bacteria helps the host plant to overcome As-induced phosphate (Pi) deficiency and consequently maintain favorable P:As ratio (Sharma et al., 2013b; Hunter et al., 2014). However, whether bacteria inoculation enhances As uptake and accumulation in plants still is a contradictory issue. It has been proposed that numerous

microbes (including fungi) can act as a barrier and limit the transfer of As into plant tissues, thereby improving growth of the host plant (Fitz and Wenzel, 2002).

Bacteria inoculation results in higher activities of the antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase, and guaiacol peroxidase) and accumulation of nonenzymatic antioxidants (carotenoids, ascorbic acid and proline)(Pandey et al., 2018). Increased concentrations of cysteine, glutathione, and non-protein thiols, and activity of glutathione S-transferase, that facilitate sequestration of As into nontoxic complexes have also been reported (Pandey et al., 2017). Thus, application of As-resistant bacteria with plant-growth-promoting traits (e.g. hormore production, siderophores, phosporus solubilization) could provide an efficiency in the phytoremediationapproach by enhancing As accumulation or by diminishing As accumulation in plants, thereby promoting higher growth, development, and yield responses with higher phytoextraction. There is also a crucial need of understanding the mechanisms involved in attenuate the toxic effects of As in As-resistant bacteria species, in order to improve the stabilization of plants in polluted sites (Coats and Rumpho, 2014; Kowalczyk and Latowski, 2018).

If the relationships that underlie plant-microbiome selection could be understood and exploited, they would be of great value for sustainable plant production (Farrar et al., 2014). It is believed that bacteria colonization is more efficient in their native host (Moliterni et al., 2012, Zhang et al., 2014), although a very interesting point would be to explore the consequences of species cross inoculation, including, for example, the inoculation of potential phytoremediation bacteria strains isolated from willow in other plant species.

1.7   BACKGROUND AND OBJECTIVES

In mostly all industrialized countries, mining activities and technological development produce a wide range of pollutants that deteriorate the environment. Besides, if these pollutants are heavy metals, the problem exacerbates since these elements can remain in the soil for thousands of years, constituting a risk for the environment and human health. Revegetation of this polluted soils has been a challenge for many years, with several trials failed to recover the original vegetation as well as to establish plant species of economic interest due to the edaphic conditions of these areas. Therefore, plant selection is probably the most important consideration to achieve a successful phytoremediation.

According to this, diverse authors (Fernandez et al. 2008; Greger and Landberg, 2009; Vangronsveld et al., 2009; Kidd et al., 2015) highlighted that in largely contaminated areas, trees are the most suitable plant type, since they can grow on marginal quality land, have massive root systems and their aboveground biomass can be harvested with subsequent resprouting without disturbing the site. Therefore, *S. atrocinerea*, a native shrub willow naturally-growing in different areas of the TE-polluted brownfield was selected and it was verified that some of these plants were able to tolerate high concentrations of As and other TEs when they were cultured *in vitro*, accumulating TEs in their tissues even at those concentrations considered toxic for plants (White and Brown, 2010). Afterwards, an *in vitro* characterization of different clones of *S. atrocinerea* was performed and the best As-accumulator was selected.

Additionally, several studies in field demonstrated that the revegetation of degraded areas with microbe inoculated trees can allow a higher survival and development rates than in trees non-mycorrhized (Fernandez-Fuego et al., 2017) or non-inoculated with bacteria (Mesa et al., 2017). In this study, a special focus was given to the bacteria population associated to roots of *S. atrocinerea* growing in the contaminated field, since same as for the plants, presence of As-resistant microbial species in the As-rich environments is highly expected due to the strong selective pressure exerted by high levels of As.

So far, most of the phytoremediation-based studies are focused on study independently the effect that bacterial communities and plants have in the uptake, degradation and accumulation of pollutants. This is usually performed in artificially contaminated soils or substrates with just one pollutant and with model plants. Consequently, taking into account a wide range of abiotic and biotic factors that cannot be monitored in the field and that could affect the phytoremediation success, it is interesting and necessary to understand plant behavior, precisely of a forestry species, together with its microbiological environment and assess the effect that this exerts in metal accumulation and tolerance in the plant. By studying aspects relative to bacterial identification and function, as well as tolerance, accumulation and chelation and stabilization of TEs by the plant, a better knowledge will be provided in the role that plant-associated bacteria play in the adaptation of plants to TE, that will be translated into an optimization of phytoremediation programs.

Therefore, the main objective of this dissertation has been to study the behavior of bacteria inoculated and non-inoculated plants growing in different experimental conditions, exposed mainly to As and other pollutants, from *in vitro* culture to field; in order to evaluate

their suitability for being used in phytoremediation. For this objective we selected a clone of grey willow (*S. atrocinerea*) able to grow naturally in an multi TE-polluted soil and the growth and TE accumulation of inoculated and non-inoculated plants was studied, as well as the detoxification mechanisms and genetic responses behind arsenic tolerance. For this purpose, five different aims have been established:

1. Identify and characterize the bacterial communities associated to roots of *S. atrocinerea* thriving in a highly polluted brownfield. By making use of computational and traditional culture methods, both non-cultivable and cultivable bacteria populations were studied, and their putative and phenotypic characterizations for tolerance to As and plant-growth-promoting traits were performed.

2. Assess the potential of *S. atrocinerea* to tolerate and accumulate As and study the mechanisms involved in these processes. At different time points, the As accumulation and speciation, analysis of thiolic compounds and genetic responses behind As tolerance were studied.

3. *In vitro* study of the effects of bacteria inoculation on As accumulation and tolerance in willow plants. Previously characterized bacteria were tested on willow plants grown *in vitro* culture and exposed to As. A detailed characterization of the physiology mechanisms behind As accumulation and tolerance was performed.

4. Characterize the physiological responses of inoculated and non-inoculated plants cultured in a polluted industrial soil. Inoculated and non-inoculated plants were grown in pots with a commercial potting substrate or a polluted industrial soil. A comparison of the plant-bacteria interaction under different substrates is done. In parallel, a six-months experiment was performed in the polluted field. The differences obtained among pot and field related to TE accumulation and tolerance are discussed.

5. Evaluate if the bacteria isolated from *S. atrocinerea* can enhance As tolerance in other plant species. Arsenic accumulation and transcriptional changes in *Arabidopsis thaliana* plants inoculated with *Pantoea* sp. were investigated. Furthermore, the role of bacteria inoculation in As tolerance under a different GSH plant supply is evaluated by the use of wild-type and *cad2-1* (GSH deficient) genotypes.

Consequently, based on the chronology of the research questions described above, this work is intended to provide a model for the rational design in the laboratory and the

subsequent effective implementation of a bacteria-assisted phytoremediation process in the field. With this in perspective and the preceding studies and considerations described throughout the introduction, the potential use of *S. atrocinerea* and its associated bacteria for the phytoremediation of the polluted soil is described as follows: in **Chapter 2**, the bacterial population associated to roots of *S. atrocinerea* is identified and characterized by cultivable and non-cultivable methodological approaches. In **Chapter 3**, different mechanisms involved in As tolerance in *S. atrocinerea* are analyzed when plants were grown *in vitro* in presence of arsenate, with an integrative study of As accumulation, speciation, PCs and gene expression. In **Chapter 4**, the effect of bacteria inoculation on these mechanisms of As tolerance and accumulation is analyzed *in vitro* culture. Apart from previous stress responses, production of phenolic compounds and hormone profile are analyzed among other parameters. In **Chapter 5**, the physiological responses of *S. atrocinerea* grown in a non-polluted substrate and an industrial-polluted substrate after six months in pots is studied. A coming from the brownfield soil was used. The physiological responses in bacteria-inoculated and non-inoculated plants are compared, as well as TEs accumulation and tolerance. Together with this, a parallel experiment in the field with non-inoculated and bacteria inoculated willow plants is described and the results are discussed and compared with those from pot conditions. In **Chapter 6**, *Pantoea* sp. is inoculated to *A. thaliana* wild-type plants as well as to GSH-deficient *cad2-1* mutant plants. In this way, further insight in the mechanisms behind As resistance, together with the role that bacteria play in the accumulation and tolerance to As is provided.

Chapter 2

Characterization and identification of the microbial populations associated to the roots of *Salix atrocinerea*

2 Characterization and identification of the microbial populations associated to the roots of *S. atrocinerea*

2.1 INTRODUCTION

Microbial processes play a major role in TE-cycling, and therefore effective phytoremediation of polluted soils involves interactions with the specific plant-associated microbes. In the same way the mechanisms of TE uptake, accumulation, exclusion, translocation, osmoregulation and compartmentalization vary with each plant species and determine its suitability for phytoremediation (Powell et al., 2002), they also vary in function of its associated microorganisms (Furini et al., 2015). For example, it is known that microbial processes or metabolites produced by soil or rhizosphere bacteria change the TE redox state and thus solubility and availability for its uptake by the plant (Kuffner et al., 2010). Besides, bacteria can produce siderophores, auxins, ACC-deaminase or solubilize phosphate. All this can improve plant growth in polluted soils and can accelerate the removal of the pollutants during the phytoremediation process (Luo et al., 2011). Indeed, an increase of plant biomass can be translated into a higher efficiency of phytoextraction (Weyens et al., 2009c).

Therefore, apart from a suitable plant species selection, the other required step for potential optimization is to focus on soil microorganisms which could accelerate this depollution process (Janssen et al., 2015; Mesa et al., 2017). One of the potentially useful approaches derived from that knowledge, together with the understanding of the interactions between microbes and the plant, is the implementation of phytoremediation techniques in combination with bioaugmentation, defined as the introduction of specific competent strains or consortia of microorganisms, in order to enhance pollutant degradation or removal from soil (Mrozik and Piotrowska-Seget, 2010).

The debate over the efficacy of delivering suitable microorganism exists for a long time (Vogel, 1996), mainly due to the difficulty in predicting the results in the field; what seems clear, however, is that when it works, the results are often very encouraging (Fernandez-Fuego et al., Mesa et al., 2017, Guarino et al., 2018). Moreover, most of the published research is focused on studying the independent effects that microbial communities and plants have on the uptake of a single pollutant, degradation and accumulation, which can be used in phytoremediation or extrapolated to other applications due to the synthesis of useful molecules in industrial biotechnoly. Therefore, in this chapter,

with the idea of enhancing phytoremediation via the use of bacteria inoculants, we explored the distribution and characterized the bacteria for properties related to TE modification and tolerance, as well as the plant-growth-promoting (PGP) traits of the microbial diversity associated with the roots of *S. atrocinerea*.

2.2   MATERIALS AND METHODS

2.2.1   *Sampling, bacterial DNA extraction, PCR amplification and high-throughput pyrosequencing*

Sa atrocinerea trees growing in different zones of the brownfield were selected and sampled. Bulk soil was taken from the proximity of trees and root samples were randomly collected from each tree and adherent rhizosphere soils from around each tree were pooled. Rhizosphere-containing soil, defined as soil in the immediate vicinity of the roots, was obtained by shaking the roots, thereby collecting the soil that was attached to the roots. Samples of four independent trees were used for the analysis of total and cultivable bacterial communities of soil, rhizosphere and endosphere (see below). Twelve DNA samples were obtained from the bulk soil, rhizosphere and endosphere (see below), using the PowerSoil DNA isolation kit (Mo Bio, USA) according to the manufacturer's instructions. The V5 to V7 region of the 16S rRNA gene was amplified using the primer set 799F (5-AACMGGATTAGATACCCKG-3) (Chelius and Triplett, 2001) and 1391R (5-GACGGGCGGTGWGTRCA-3) (Walker and Pace, 2007). The primers 799F and 1391R were selected to minimize chloroplast contamination by providing considerable mismatches with chloroplast sequences (<0.1% of total sequences retrieved) (Beckers et al., 2016). Since the concentration of bacterial DNA in comparison with the plant DNA was low, we chose a nested PCR strategy to amplify the samples. A first round of PCR amplification was conducted using primers without the Roche 454 pyrosequencing adaptors and sample-specific barcode. Products obtained from triplicate PCR reactions were pooled and purified using the QIAquick PCR purification kit (Qiagen Benelux B.V., the Netherlands), the quality of the amplicon pools was evaluated using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Belgium), and DNA concentration of amplicon libraries was determined using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, CA, USA) and a Fluostar Omega plate reader (BMG Labtech, Germany) and pooled in equimolar concentrations.

Each 25 μL PCR mixture contained 10 ng of DNA and PCR was performed using the FastStart high-fidelity PCR system (Roche Applied Science, Mannheim, Germany), according

to the instructions provided by the manufacturer. Cycling conditions included initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 1 min; a final extension phase was conducted at 72 °C for 10 min. PCR amplicon pools were cleared from residual primers and primer dimers by separating the PCR products on a 1.5% agarose gel. Bacterial amplicons were excised from the gels using the QIAquick gel extraction kit (Qiagen Benelux N.V., The Netherlands). The amplicon length of sequences produced by primer set 799F-1391R was reduced by amplifying the samples with primer set 967F (5-CAACGCGAAGAACCTTACC-3) and 1391R in a second round (Sogin et al., 2006). The forward primer was fused to the Roche 454 pyrosequencing adaptor A and a sample-specific 10-bp barcode (multiplex identifiers [MIDs]), and the reverse primer was fused to adaptor B (Roche Applied Science, Germany). The PCR cycling conditions were identical to the ones previously described, with the exception of the number of PCR cycles, which was lowered to 25. Sequencing was carried out on one-eighth of a picotiter plate on a Roche Genome Sequencer FLX using Titanium chemistry (Roche Applied Science, Germany) by LGC Genomics (Germany).

2.2.2   *Isolation and phylogenetic affiliation of bacterial strains*

Since the aim of this study is to use the most promising bacteria for inoculation experiments, the cultivable bacterial community of rhizosphere and endosphere was further investigated. To obtain the rhizospheric bacteria, 5 g of rhizosphere soil was shaken in 10 ml of 0.1% sodium pyrophosphate (Sigma-Aldrich, USA). Soil particles were allowed to settle for 1 h. The supernatants were diluted to 10^8 cfu (colony forming units) mL^{-1} and plated on two different media, 1:10-diluted tryptic soy agar (TSA) (Janssen et al., 2002) and 1:10-diluted 869 solid medium (Eevers et al., 2015). The plates were incubated for 7 days at 30°C. The remaining supernatants were stored at – 80 °C until DNA extraction. To get the cultivable root endophytes, roots were rinsed under running tap water, surface sterilized for 10 min in 2% active chlorine solution supplemented with one droplet of Tween 80 (Merck, Germany) per 100 ml of solution, and subsequently rinsed three times for 1 min in sterile distilled water. Next, root samples were macerated in 10 ml of 10 mM $MgSO_4$ using pestle and mortar. The extracts obtained from the macerated roots were serially diluted to 10^5 cfu mL^{-1}, plated on the same medium, and incubated 1h at 30°C. Colonies with different morphotypes were selected and repeatedly streaked until axenic cultures were obtained (Weyens et al., 2009a). Of each morphotype, 1 to 10 replicates were purified and stored in a glycerol solution, NaCl:glycerol [0.85% (w:v)] at – 80 ˚C.

From all purified bacterial strains total genomic DNA was extracted using the

DNeasy® Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Polymerase chain reaction (PCR) amplification of 16S rRNA, amplified 16S rRNA restriction analysis (ARDRA) and gel electrophoresis were performed according to Weyens et al. (2009a). Bacterial strains with the same ARDRA patterns were grouped and the purified PCR product (QIAquick 96 PCR Purification Kit (Qiagen, Valencia, CA, USA)) of one representative isolate of each group was sequenced by Macrogen (Seoul, Korea) using an Automatic Sequencer 3730XL. Consensus sequences were obtained with Geneious Basic 5.3.6 and sequence matches were searched for on the Ribosomal Database Project II (http://rdp.cmcme.msu.edu/seqmatch/seqmatch intro.jsp) and the database of the National Center for Biotechnology Information (http://rdp.cme.msu.edu/seqmatch/seqmatchintro.jsp). The phylogeny of the rhizospheric and endophytic cultivable bacteria associated with *S. atrocinerea* were reconstructed using partial 16S rRNA gene sequences. Sequences were aligned using ClustalW (Thompson et al., 1994) and phylogenetic analyses were conducted with MEGA v7 (Kumar et al., 2016). The evolutionary distances were inferred using the Kimura-2 parameter model. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL), and then selecting the topology with superior log likelihood value.

2.2.3   *Sequencing data analysis and adscription of functional properties*

16S rRNA gene amplicon sequences were analyzed using Quantitative Insights into Microbial Ecology (QIIME) package (version 1.9.0) (Caporaso et al., 2010b). First, reads were assigned to samples based on their nucleotide barcode. This step also performed quality filtering (minimum quality score of 30, eliminating reads with 200 bp lengths and 0 ambiguous bases and mismatches in primer sequences). Chimeric sequences were checked and removed using USEARCH (Edgar et al., 2011), and the resulting sequences obtained were clustered into operational taxonomic units (OTUs) using a minimum identity of 97% based on their similarity according to UCLUST (Edgar, 2010). Representative sequences from each OTU were aligned using PyNAST (Caporaso et al., 2010a) and singletons were excluded from the analysis. Sequences classified as chloroplasts (0.3%) or mitochondria (0.5%) were removed from the alignment. Taxonomic annotation was performed through comparison against the Greengenes database (release May, 2013) for the 16S rRNA gene amplicon data.

All calculations and statistical analyses for the microbial community were carried out in R version 1.1.453 (R Development Core Team, 2016). Analyses and visualizations were performed using ggplot2 version 2.2.1 (Wickham, 2009). Alpha diversity was calculated

through observed species (observed OTUs), richness estimator (Chao1), and diversity indices (Shannon and Simpson) to compare the diversity of the bacterial community within samples. Beta diversity was evaluated through principal coordinate analysis (PCoA) of weighted and unweighted UniFrac and Bray–Curtis distances. Alpha- and beta diversity were estimated using the package phyloseq (version 1.24.0) (McMurdie and Holmes, 2013). Rarefaction curves were generated using the vegan package (version 2.5-1) (Oksanen et al., 2017). Non-parametric analyses of variance on 16S data were performed to evaluate differences between bacterial communities with permutation based hypothesis tests, ANOSIM (namely analysis of similarities), and adonis (permutational multivariate analysis of variance) with 99 and 999 permutations, respectively (Fierer et al., 2010). A linear discriminant analysis effect size (LEfSe) was used to determine which genera were significantly different based on environment type. Significantly different (P < 0.05) taxa among groups of samples are identified by LEfSe using the Kruskal-Wallis test and the effect size of each of these is estimated using linear discriminant analysis (LDA) (Segata et al., 2011). An LDA score of 3.0 was used as the cut-off for plotting differentially abundant genera.

Metagenome functional content from 16S rRNA was predicted using PICRUSt version 1.1.3 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013). QIIME biological observation matrices were imported into PICRUSt, and metagenomes inferred using the proposed pipeline. The accuracy for the predicted metagenome was tested through the Nearest Sequenced Taxon Index (NSTI), reflecting the presence of reference genomes that are closely related to the samples in analysis (Langille et al., 2013). Using MicrobiomeAnalyst (Dhariwal et al., 2017), the gene abundance data were normalized by cumulative sum scaling (CSS). Heatmaps were generated with the normalized metabolic pathway abundances using the Euclidean distance method for endosphere, bulk and rhizosphere soil microbiomes.

2.2.4 *Plant-growth-promoting (PGP) traits of bacterial strains*

All purified bacterial strains were screened for their potential PGP traits (production of indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity; ACCD), nutrient uptake enhancing properties (production of siderophores, organic acids, volatile compounds (such as acetoin), nitrogen fixation, phosphorous solubility) and As tolerance. Results were assessed qualitatively. Before screening, strains were grown in 869 medium and subsequently washed 2 times with 10 mM $MgSO_4$. Isolates that were not able to grow in the different test media for PGP at 30 ˚C were considered as not detectable.

Media without cell suspension served as control. The PGP characteristics were screened as described previously by Croes et al. (2013). To test As tolerance, the isolates were plated on selective 284 medium with a carbon mix (Schlegel et al., 1961) and 0, 25, 50 and 100 mM As V (added as Na_2HAsO_4 $7H_2O$, Sigma-Aldrich, USA) or 0, 10 and 20 mM As III (added as $NaAsO_2$, Sigma-Aldrich, USA). After an incubation period of 7 days at 30 ˚C, growth of the isolates was rated by sight.

2.3 RESULTS

2.3.1 Diversity of total bacterial community from soil, rhizospheric and endophytic fractions

Bacterial DNA was isolated from bulk soil, rhizosphere and endosphere of *S. atrocinerea* and 16S rRNA genes were amplified, sequenced by 454 pyrosequencing and analyzed bioinformatically. After quality screening and denoising, we obtained a total of 16,073; 101,676 and 100,591 high-quality sequences from endosphere, rhizosphere and

Table 2.1. Pyrosequencing data and goods coverage obtained from the bulk soil, rhizosphere and endosphere of *S. atrocinerea.*

Sample		Nº of sequences	Nº of trimmed sequences[a]	Nº of OTUs	Goods coverage
Soil	Soil1	29838	15526	1199	0.97
	Soil2	18848	11991	914	0.97
	Soil3	29643	12866	1037	0.97
	Soil4	22262	5317	991	0.94
Rhizosphere	SR1	17367	9288	590	0.98
	SR2	35828	20164	1029	0.98
	SR3	24416	15713	659	0.98
	SR4	24065	10508	636	0.98
Endosphere	SE1	4927	2888	255	0.97
	SE2	4938	3164	160	0.98
	SE3	3591	1708	336	0.94
	SE4	2617	1988	82	0.98

[a] Reads after trimming and chimera removal.

bulk soil, respectively, with an average read length of 421 bp. The number of operational taxonomic units (OTUs) based on a 97% similarity threshold was smaller in the endosphere than in the rhizosphere for all trees sampled (82 to 336 compared to 636 to 1029 respectively). In the bulk soils the OTUs were among 914-1199) (**Table 2.1**).

Among trees, microbial communities exhibited greater diversity in the bulk soil (average number of observed OTUs, Chao1, Shannon index, and Simpson index were 1,035;

1,148; 4.2 and 0.84, respectively) than in the rhizosphere (average number of observed OTUs, Chao1, Shannon index, and Simpson index were 728; 800; 3.85 and 0.9, respectively) and the endosphere (average number of observed OTUs, Chao1, Shannon index, and Simpson index were 186; 255; 2.73 and 0.63, respectively) (**Fig. 2.1A**). Rarefaction curves revealed a lower diversity in the root endosphere. In the current analysis, the asymptotic shape of the curves indicates that sequencing depth was sufficient to capture the entire bacterial diversity (**Fig. 2.1B**).

A **B**
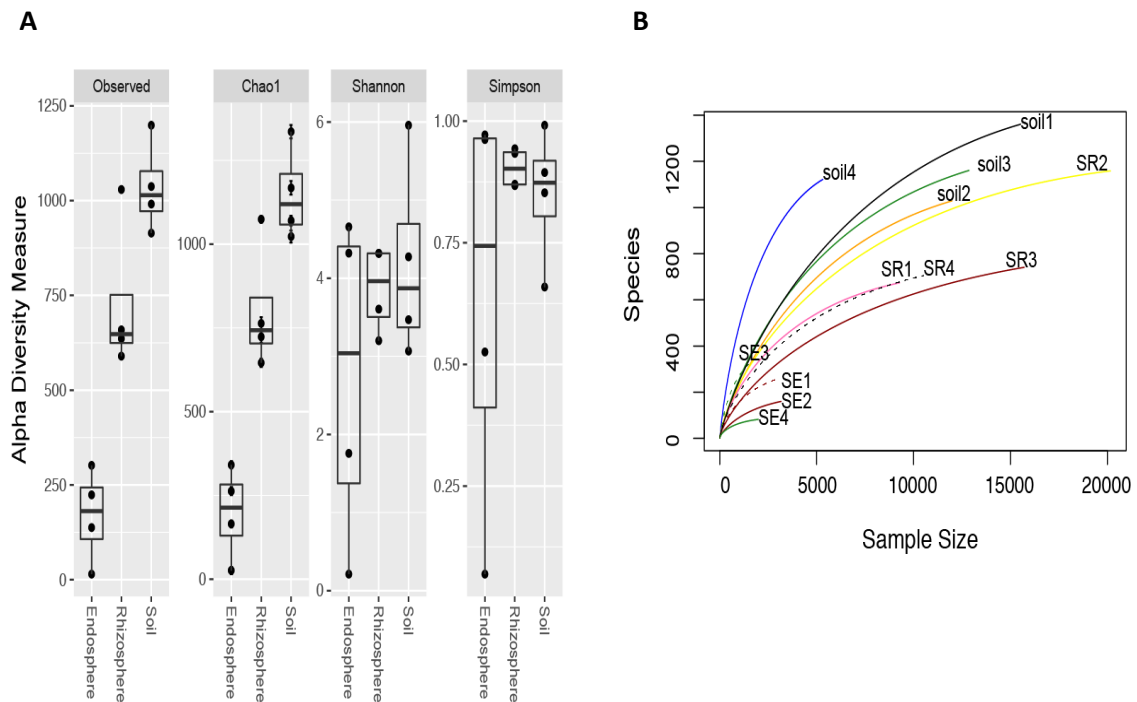
Fig 2.1. (A) Values of several alpha diversity metrics for the endosphere (SE), rhizosphere (SR) and bulk soil (soil) microbial populations of 4 trees of *S. atrocinerea*. Boxes show the inter-quartile range for the three communities. **(B)** Rarefaction curves at the 3% distance cutoff microbial communities.

To visualize overall differences in the structures of the microbial communities between samples, phylogenetic tree-based beta diversity metrics (UniFrac) and pairwise Bray-Curtis were calculated and ordinated in two-dimensional PCoA plots. PCoA analyses demonstrated that the rhizosphere and bulk soil samples clustered together, whereas the samples from the endosphere comprised another group. The overall variances explained by coordinate 1 and 2 is higher in weighted UniFrac (~47%), compared to unweighted (~34%) and Bray-Curtis (~30%) (**Fig. 2.2 A-C**).
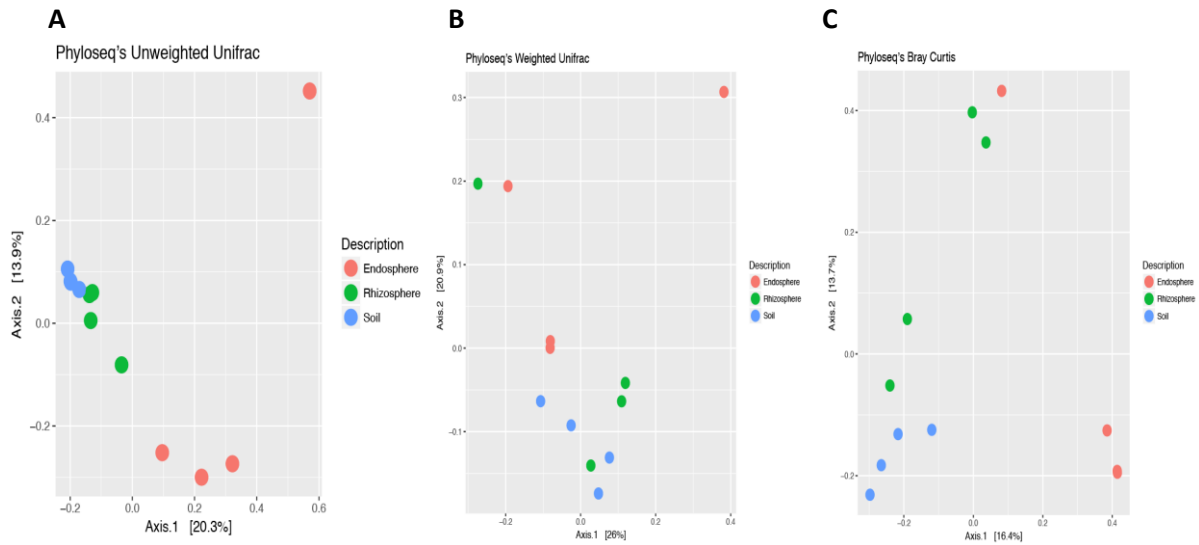
Fig 2. Principal coordinates analysis (PCoA) derived from pairwise unweighted Unifrac distances **(A)**, weighted Unifrac distances **(B)** and Bray–Curtis distances **(C)** of 16S rRNA gene between microbial communities of bulk soil (blue circles), rhizosphere (green circles) and endosphere (pink circles) populations.

To statistically support the visual clustering of the bacterial communities in the PCoA analyses, we compared DNA-based samples by means of permutation-based hypothesis tests: analysis of similarities (ANOSIM), and permutational multivariate analysis of variance (adonis). The non-parametric analyses of variance on 16S data confirmed that the composition of the three communities was significantly different (ANOSIM: p = 0.01, R2 = 47%; adonis: p = 0.001, R2 = 25%). From the classifiable sequences, the bacterial community composition was analyzed at three different taxon levels (phylum, order and genus), considering abundant OTUs to be the sequences present in more than one sample and representing more than 1% of the total sequences. At the phylum level, five bacterial phyla were overrepresented and the willow microbiome was mainly affiliated to the phyla Proteobacteria (41.4%), Actinobacteria (21.1%), Firmicutes (13%), Candidate division TM7 (9.4%) and Bacteroidetes (8.9%) (**Fig. 2.3A**).
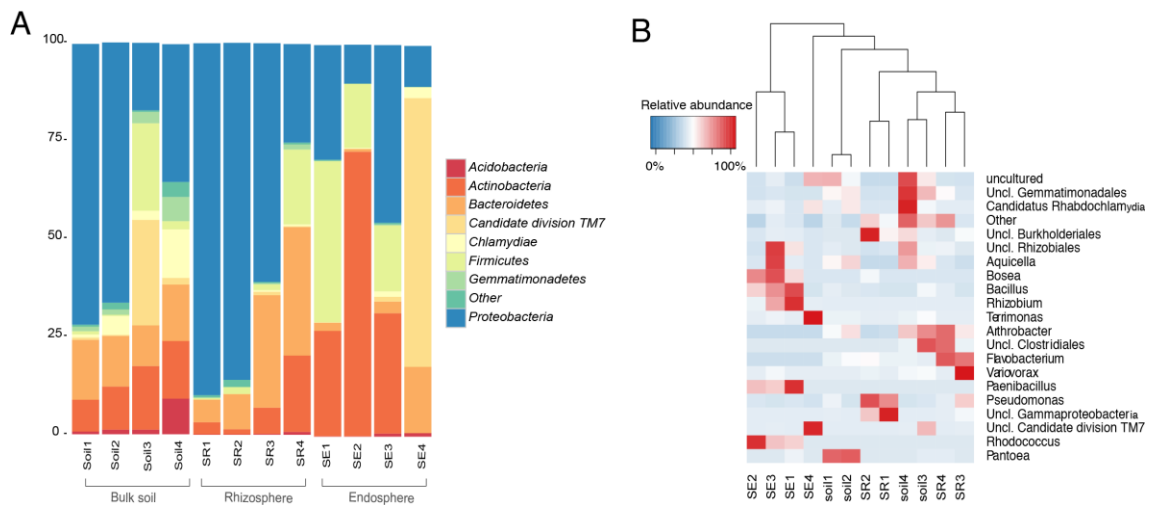
Fig 2.3. Microbial community analysis plots based on 16S rRNA gene pyrosequencing in endosphere and rhizosphere. Bar chart showing the relative abundance of major bacterial phyla (over 1%)**(A)**. Heatmap showing the distribution of bacterial order and genus level (over 1%)**(B)**.

A closer inspection revealed that genus *Pseudomonas* was predominant in the rizosphere in SR2 (55%), SR1 (40.7%), and SR3 (24.3%). The endosphere displayed a higher proportion of *Rhodococcus* (70.8% SE2, 27.5% SE3, and 23.5% SE1). The soil was enriched in *Pantoea* (58.8% Soil 2 and 56.6% Soil 1). The bulk and rhizosphere soils shared similar order- and genus-level profiles, with *Arthrobacter* and Uncl. *Clostridiales* being the most abundant. However, the root endosphere was enriched with *Bosea*, *Bacillus*, *Rhizobium*, *Paenibacillus* and *Rhodococcus*, which was in big contrast to the communities of the bulk and rhizosphere soils (**Fig. 2.3B**).

Linear discriminant analysis effect size (LEfSe) was used to determine which genera were enriched in the three compartments. *Rhodococcus* was the genus most enriched in the endosphere, *Pantoea* in the bulk soil and *Pseudomonas* in the rhizosphere (**Fig. 2.4**).
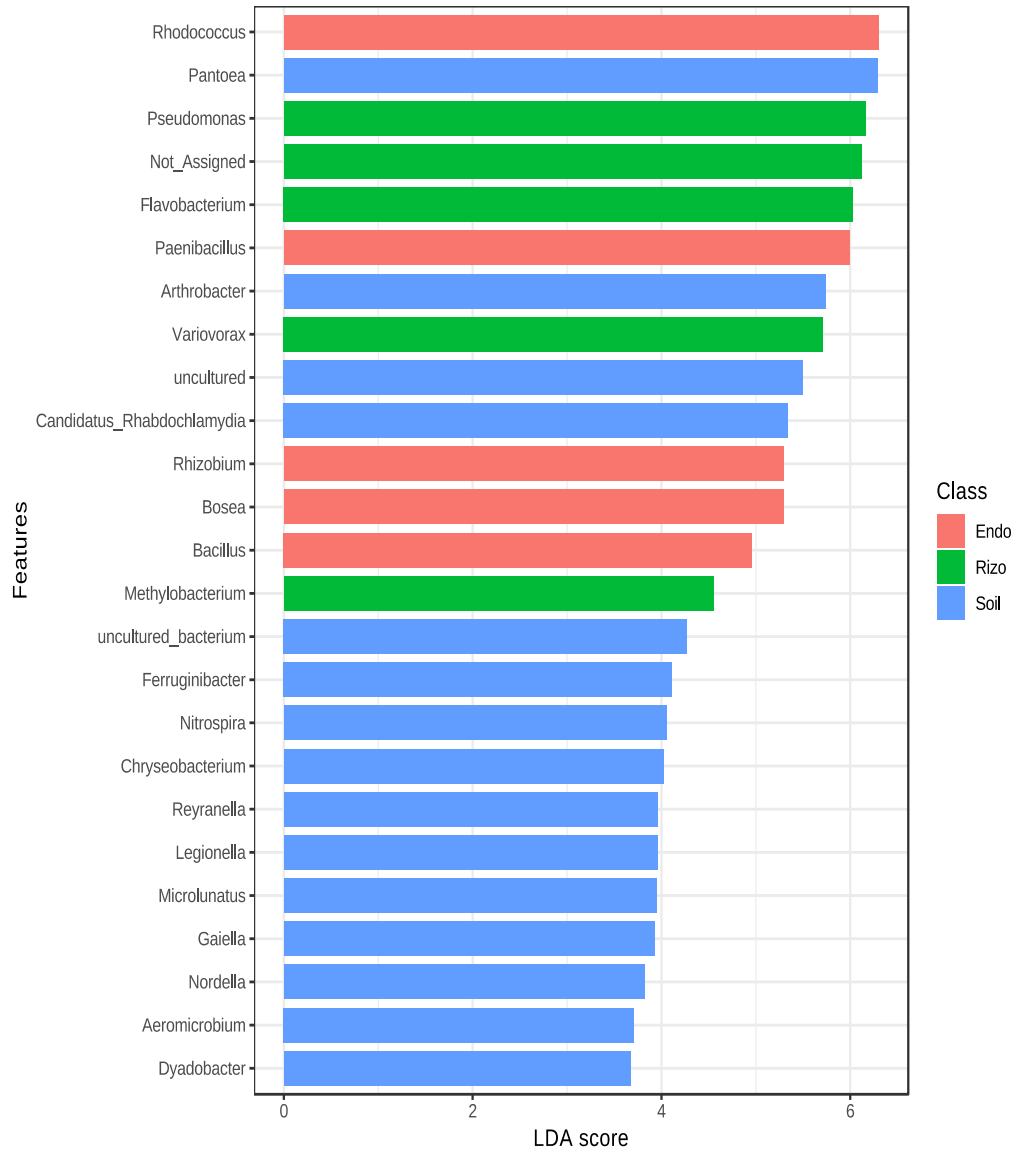
Fig 2.4. Differentially abundant bacterial taxa in each of the three environment types, as assessed using linear discriminant analysis (LDA) with effect size measurements (LEfSe). The genera enriched with a LDA score greater than 1.0 are displayed. The bar graph showing the LDA scores of top 25 significant features. Endo = Endosphere, Rizo = Rhizosphere, Soil = Bulk soil.
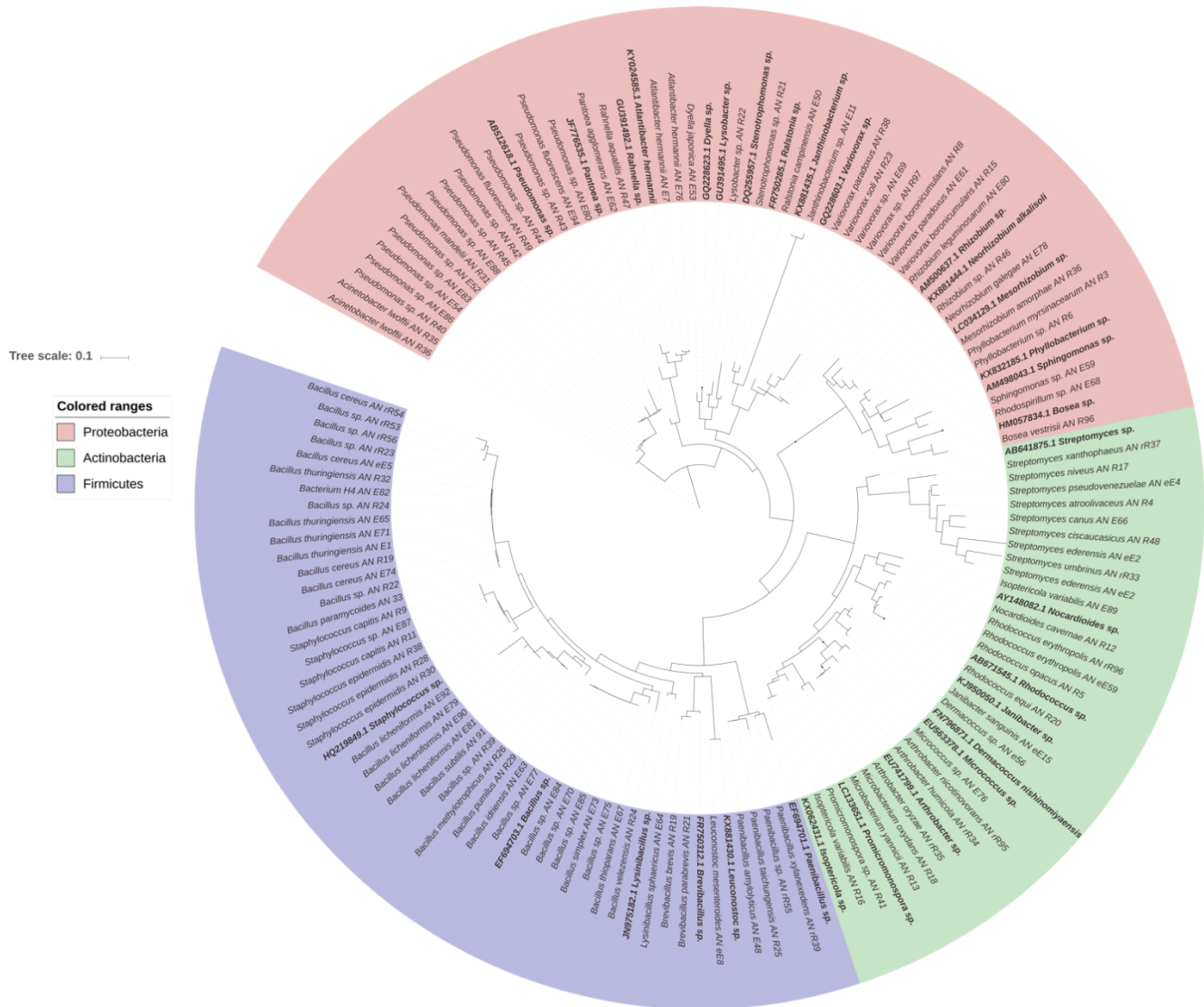
Fig 2.5. Phylogenetic correlations of endophytic and rhizospheric strains isolated from *S. atrocinerea* roots (endosphere, n = 65; rhizosphere, n = 71). Neighbor-joining tree inferred using MEGA version 7.0. Previously species associated with rhizosphere and roots from databank were used as reference strains; the numbers to the left of the organism names are their accession numbers. The scale bars indicate the number of substitutions per nucleotide, 372 substitutions per site.

2.3.2   *S. atrocinerea-associated bacteria*

A total of 136 bacterial strains (65 endophytic and 71 rhizospheric) were isolated as axenic cultures (**Table 2.2**). The phylogeny of the cultivable bacteria associated with *S. atrocinerea* (based on the 16S rRNA genes) showed a clear distribution into the strongly predominant phyla: Proteobacteria, Firmicutes, and Actinobacteria (**Fig. 2.5**). Each of these phyla accounted for 37.25, 45, and 17.64 % of endophytic strains, respectively, and 33.9, 38.9, and 27.11 % of rhizospheric strains, respectively. Twenty-seven and eighteen different genera were obtained from the endosphere and rhizosphere, respectively. *Bacillus* (30%) and *Pseudomonas* (14%) predominated in endophytes while *Bacillus* (27%) and *Streptomyces* (12%) were the most abundant in the rhizosphere (**Fig. 2.6**).
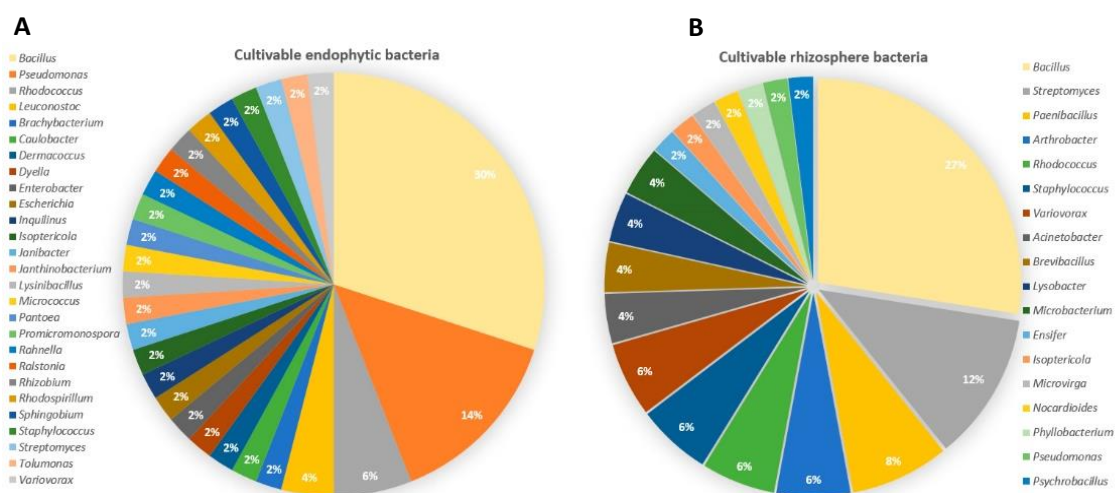
Fig 2.6. Percentage of different genera representing the endophytic (**A**) and rhizospheric (**B**) bacteria isolated from *S. atrocinerea* roots (endosphere, n = 65; rhizosphere, n = 71).

2.3.3   *Comparative prediction of the functional proteins of the total microbial communities and characterization of cultivable bacterial strains*

To obtain information about the metabolic characteristics of the bacterial communities that might be involved in the uptake and translocation of TEs in *S. atrocinerea*, the functional composition of the microbial metagenomes was predicted from amplicon data of the 16S profile by using the PICRUSt pipeline (Langille et al., 2013). The low values of Nearest Sequenced Taxon Index (NSTI), indicate a close mean relationship with sequenced genome representatives. Samples had good mean NSTI values (bulk soil = 0.109 ± 0.02, rhizosphere = 0.095 ± 0.02 and endosphere = 0.064 ± 0.02).

The PICRUSt model allowed to infer proteins related to the resistance to As, especially those involved in the decrease of transport of arsenate and arsenite, were detected in higher amounts in the rhizosphere than in the bulk soil. The same was the case for proteins

hypothetically involved in TE efflux systems (HME) of the RND superfamily (Nies, 2003; Du et al., 2015), zinc transport system and proteins related with ABC-transport systems. A more balanced representation in soil and rhizosphere was revealed for proteins related to the GSH metabolism and aquaporins. Interestingly, proteins hypothetically related with N fixation were well represented in endophytes. Finally, a diverse flagellar-related proteins were present in the bulk soil and to a lower extent in the rhizosphere strains (**Fig. 2.7**).

More direct information on the beneficial functional characteristics of the isolated strains was obtained by direct *in vitro* evaluation of their capacity to produce one or more PGP traits and their resistance to As. Endophytic bacteria showed a greater capability for N fixation (**Table 2.4**), whereas ACCD, siderophore and acetoin production were more present in rhizosphere bacteria (**Table 2.4**). All strains showed high resistance to As III and As V (**Table 2.4**) and the six isolates that showed the highest numbers of PGP traits were tested for their capability to reduce As V to As III. From the endosphere strains *Rahnella aquatilis, Pantoea* sp. and *Pseudomonas fluorescens*, only *Pantoea* sp. could reduce 1mM As V to As III and in less than 48 h (**Fig. 2.8**). All rhizosphere isolates (*Rhodococcus erythropolis*, *Paenibacillus taichugensis* and *Arthrobacter nicotinovorans*), showed As V reduction to As III, with *R. erythropolis* exhibiting the fastest reduction time (**Fig. 2.8**). Two isolates were finally selected, *i.e.* the phosphorus solubilizing, nitrogen-fixing, IAA-, ACCD-, organic acids-, siderophore-, and acetoin-producing *R. erythropolis* strain, and the phosphorus solubilizing, nitrogen-fixing, IAA-, ACC deaminase- and siderophore-producing *Pantoea* sp. strain (**Table 2.4**). These bacteria were tested subsequently for their potential capability to improve phytoremediation of the multi-TE polluted soil.
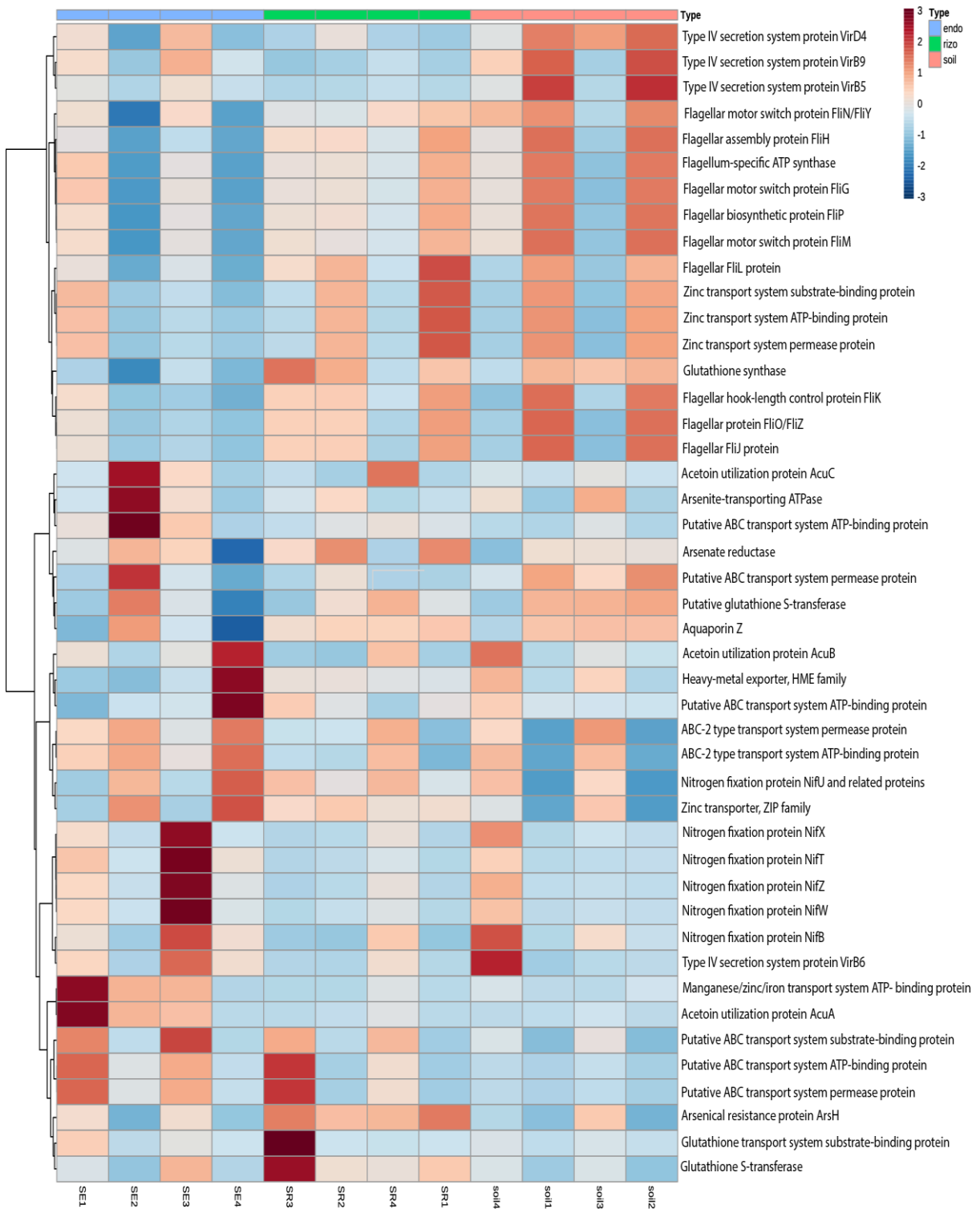
**Fig. 2.7.** KEGG differential protein presence in the different microbiomes.

Table 2.3. Phylogenetic affiliation and plant growth-promoting traits (N fix = nitrogen fixation; ACCD = production of 1-aminocyclopropane-1-carboxylate deaminase; P = phosphate solubilization; OA = production of organic acids; SID = production of siderophores; Acet = production of acetoine and IAA = production of indole-3-acetic acid) of cultivable endophytic and rhizospheric bacteria associated to *S. atrocinerea*. Phylogenetic affiliations were based on 16S rRNA gene sequences of about 800 bp length. Strains in boldface were selected for further analysis. *In vitro* detection of the trait is indicated by a (+).

Isolate	Plant Growth-Promoting traits							As Resistance MIC (mM)	
	N fix	ACCD	P sol	OA	SID	Acet	IAA	As III	As V
Endosphere									
Bacillus cereus AN_eE5			+			+		5	5
Bacillus cereus AN_eE12		+				+	+	5	5
Bacillus cereus AN_e74					+			>10	5
Bacillus idriensis AN_e63	+				+			>20	50
Bacillus licheniformis AN_e79	+			+	+	+		5	5
Bacillus licheniformis AN_e81	+					+		5	<5
Bacillus licheniformis AN_e92	+			+	+	+		5	5
Bacillus licheniformis AN_e90		+				+		<5	<5
Bacillus amyloliquefaciens AN_e95	+	+		+	+	+		5	5
Bacillus mycoides AN_e72	+							>20	>100
Bacillus simplex AN_e73	+				+			<5	<5
Bacillus simplex AN_e78	+			+				>20	50
Bacillus sp. AN_e70	+							<5	5
Bacillus sp. AN_e75								5	10
Bacillus sp. AN_e77					+		+	>20	>100
Bacillus sp. AN_e84								<5	5
Bacillus sp. AN_e85	+							<5	50
Bacillus sp. AN_e93	+			+		+		<5	<5
Bacillus subtilis AN_e91		+	+	+	+			>20	>100
Bacillus thioparans AN_e67					+			<5	<5
Bacillus thuringiensis AN_eE1	+		+		+	+		>20	>100
Bacillus thuringiensis AN_e65	+							<5	<5
Bacillus thuringiensis AN_e71	+		+			+		<5	<5
Bacterium H4 AN_e82					+	+		5	<5
Brachybacterium paraconglomeratum AN_eE6	+	+				+	+	5	>10
Dermacoccus sp. AN_e56							+	<5	<5
Dyella japonica AN_e53	+				+		+	>20	>100
Atlantibacter hermannii AN_eE7		+					+	5	5
Atlantibacter hermannii AN_e76E			+		+	+	+	5	5
Inquilinus ginsengisoli AN_eE10			+		+	+		5	5
Isoptericola variabilis AN_e89	+	+			+			>20	>100
Janibacter sanguinis AN_eE3	+				+		+	5	>100
Janibacter sanguinis AN_eE15			+			+	+	>20	>100
Janthinobacterium sp. AN_eE11			+		+	+	+	5	5
Leuconostoc mesenteroides AN_eE8	+						+	5	10
Leuconostoc mesenteroides AN_eE13	+		+	+		+	+	5	5
Lysinibacillus sphaericus AN_e64								>20	50
Micrococcus sp. AN_e76		+					+	5	10
Pantoea sp. AN_e62	+	+	+	+	+		+	>20	50
Pseudomonas fluorescens AN_e94	+	+			+	+		>20	50
Pseudomonas sp. AN_e52		+	+		+		+	5	50
Pseudomonas sp. AN_e54	+		+		+			>20	>100
Pseudomonas sp. AN_e80	+	+	+		+	+	+	>20	>100
Pseudomonas sp. AN_e83					+		+	>20	>100
Pseudomonas sp. AN_e86	+		+		+		+	>20	>100
Pseudomonas sp. AN_e88		+	+		+		+	5	50
Ralstonia campinensis AN_e50		+			+		+	<5	5
Rahnella aquatilis AN_r47	+	+	+	+	+	+	+	>20	50
Rhizobium leguminosarum AN_eE80	+					+		5	5
Neorhizobium galegae AN_eE78	+		+	+	+	+		5	5
Rhodococcus erythropolis AN_eE59	+	+	+				+	5	5
Rhodococcus sp. AN_e55	+				+			>20	>100
Rhodococcus sp. AN_e57					+	+	+	>20	>100
Rhodospirillum sp. AN_e68			+	+	+			>5	50
Sphingomonas sp. AN_e59					+			5	<5
Staphylococcus sp. AN_e87	+							<5	<5
Streptomyces canus		+						<5	5
Streptomyces ederensis AN_eE2					+		+	5	>100
Streptomyces phaeochromogenes AN_eE14	+		+	+		+	+	>20	>100
Streptomyces phaeochromogenes AN_eE60	+		+			+		5	5
Streptomyces ederensis AN_e51		+			+			>20	50
Streptomyces prunicolor AN_eE9			+		+			5	5
Streptomyces pseudovenezuelae AN_eE4								5	5
Streptomyces pseudovenezuelae AN_e77E			+		+			<5	<5
Variovorax paradoxus AN_e61					+			>20	>100
Variovorax sp. AN_e69					+		+	<5	10
Total	32	17	23	11	38	24	25		
%	21	11	15	7	25	16	17		

	N fix	ACCD	P sol	OA	SID	Acet	IAA	As III	As V
Rhizosphere									
Bacillus sp. AN_rR22		+			+			>20	50
Bacillus sp. AN_rR53	+		+		+	+		>20	>100
Acinetobacter lwoffii AN_r36	+	+			+			>20	>100
Acinetobacter lwoffii AN_r35					+			5	<5
Nocardioides cavernae AN_r12		+			+	+		5	5
Arthrobacter oryzae AN_rR35		+	+				+	<5	>100
Arthrobacter oryzae AN_rR40	+	+		+	+	+	+	<5	>100
Arthrobacter humicola AN_rR34					+	+	+	10	>100
Arthrobacter nicotinovorans AN_rR95	+			+	+	+	+	>20	>100
Bacillus amyloliquefaciens AN_r37						+		>20	5
Bacillus cereus AN_rR19		+	+		+	+	+	5	5
Bacillus cereus AN_rR20						+		5	5
Bacillus paramycoides AN_r33						+		5	<5
Bacillus cereus AN_rR54			+		+	+	+	>20	10
Bacillus sp. AN_rR24		+			+	+		>20	10
Bacillus methylotrophicus AN_r26					+	+		5	<5
Bacillus pumilus AN_r29				+	+	+		5	<5
Bacillus velezensis AN_r24	+					+		<5	5
Bacillus sp. AN_r1			+		+			5	50
Bacillus sp. AN_r39					+	+		5	5
Lysobacter sp. AN_r22						+		>20	50
Bacillus sp. AN_rR23	+					+		>20	10
Bacillus sp. AN_rR56	+		+		+	+	+	5	5
Bacillus thuringiensis AN_r32			+			+		<5	<5
Bosea vestrisii AN_r96	+	+			+			>20	50
Brevibacillus brevis AN_r19								>20	5
Brevibacillus parabrevis AN_r21					+	+		>20	5
Sinorhizobium sp. AN_r14		+	+		+			5	10
Isoptericola variabilis AN_r16	+	+			+			>20	>100
Mesorhizobium amorphae AN_rR36		+	+		+	+	+	<5	<5
Microbacterium yannicii AN_r13				+	+	+		5	50
Microbacterium oxydans AN_r18	+	+			+			>20	50
Paenibacillus taichungensis AN_r25		+			+			>20	50
Paenibacillus sp. AN_r34					+			<5	10
Paenibacillus xylanexedens AN_rR39	+				+	+		>20	>100
Paenibacillus sp. AN_rR55	+		+	+	+	+	+	>10	10
Phyllobacterium myrsinacearum AN_r3		+	+		+	+		<5	50
Phyllobacterium sp. AN_r6		+	+		+			>20	>100
Promicromonospora sp. AN_r41								>20	10
Pseudomonas mandelii AN_r31		+	+		+		+	5	50
Pseudomonas fluorescens AN_r49		+	+	+	+		+	>20	50
Pseudomonas sp. AN_r40		+	+		+			5	>100
Pseudomonas sp. AN_r42		+	+	+	+		+	>20	50
Pseudomonas sp. AN_r43		+	+	+	+		+	>20	50
Pseudomonas sp. AN_r44		+	+	+	+			>20	50
Pseudomonas sp. AN_r45	+				+		+	5	50
Psychrobacillus sp. AN_r27							+	5	<5
Rhizobium sp. AN_r46	+	+	+		+			>20	>100
Rhodococcus equi AN_r20		+			+			>20	50
Rhodococcus erythropolis AN_rR96	+	+	+		+	+	+	>20	>100
Rhodococcus opacus AN_r5					+	+		>20	50
Staphylococcus capitis AN_r9					+			5	<5
Staphylococcus capitis AN_r11	+				+			<5	5
Staphylococcus epidermidis AN_r2			+		+			>20	>100
Staphylococcus epidermidis AN_r28				+	+	+	+	<5	10
Staphylococcus epidermidis AN_r30	+	+			+		+	>20	>100
Staphylococcus epidermidis AN_r38		+						>20	>100
Stenotrophomonas sp. AN_rR21	+	+	+			+	+	<5	>100
Streptomyces atroolivaceus	+	+			+			>20	50
Streptomyces aurantiacus AN_r7		+			+		+	>20	50
Streptomyces niveus AN_r17		+						5	<5
Streptomyces ciscaucasicus	+	+			+			>20	50
Streptomyces umbrinus AN_rR33	+		+		+	+	+	<5	5
Streptomyces xanthophaeus AN_rR37	+		+		+	+		>10	10
Variovorax paradoxus AN_rR38			+		+		+	5	10
Variovorax boronicumulans AN_r8				+	+			<5	5
Variovorax boronicumulans AN_r15					+	+		<5	5
Variovorax soli AN_r23		+			+			5	5
Variovorax sp. AN_rR97	+		+		+			5	5
Xanthomonas sp. AN_r10					+			>20	>100
Total	24	33	27	12	57	33	23		
%	17	23	19	9	40	23	16		

Oxic bacterial As V reduction
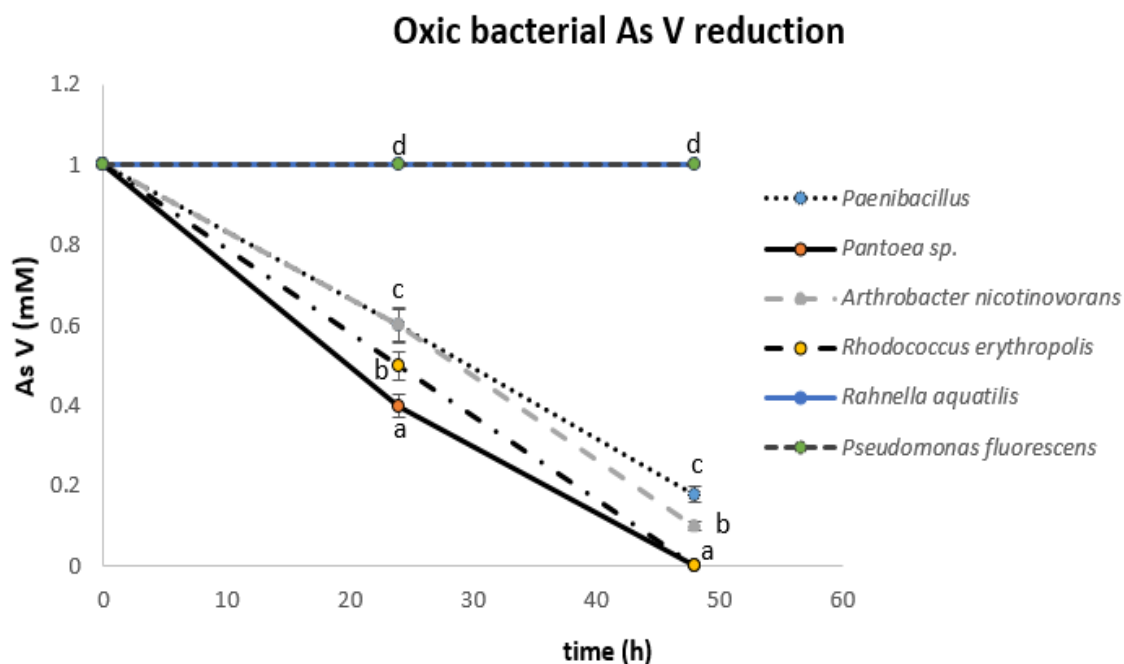
Fig. 2.8. Arsenate reduction (mM) from the six bacteria isolates associated to *S. atrocinerea* showing the greatest number of plant growth-promoting traits grown in 284 medium for 48 h under 1mM As V. Different letters at time point among strains denote significant differences on HSD test at p < 0.05.

2.4 DISCUSSION

The contribution of the plant-associated microbial populations (rhizosphere and even endosphere) is crucial in phytoremediation (Yadav et al., 2018). A profound knowledge of plant-microorganism interactions is therefore of great practical value to improve the efficiency of phytoextraction (Kong and Glick, 2017b). This also has been exemplified in this work through the use of plant growth-promoting bacteria to enhance phytoremediation. Thus, in addition to knowing the physical and chemical characteristics of the soil (see below), the exploration of the plant-associated microbial diversity, its distribution and function, and subsequently, the selection of the most appropriate microorganisms for the implementation of a microorganism-assisted phytoremediation is very important (Ashraf et al., 2017).

Studies on the microbiome of different plant species suggest that root exudates contribute to shaping the microbial community structure and therefore the plant–microbe interactions (Hartmann et al., 2009; Sasse et al., 2018). Thus, each plant species, and more precisely every genotype, has a strong effect on microbial community composition since plant natural variation in root exudates leads to distinctive microbial communities (Jacoby et al., 2017). Also, the higher variety of biotic interactions occurring in the rhizosphere, as

roots with mycorrhizal fungi and root pathogens, root-bacteria interactions, interactions within the microbial community, and interactions of mesofauna with the microbial community, can affect the diversity and composition of the microbial community associated with roots (Hawkes et al., 2007; Nihorimbere et al. 2011). The results of the sequence analysis of the total bacterial communities in our work showed differences in the composition of bacterial taxa in bulk soil and plant rhizosphere, relative to the endophytic fraction. These results indicate that plants select for specific bacterial taxa and thus exert some control over their microbiomes (Bulgarelli et al., 2012; Turner et al., 2013; Zgadzaj et al., 2016).

Methodological approaches are now available that allow to infer the complex metabolic interactions that take place between the total microbial populations (including the uncultivable) (Kong and Glick, 2017a), and the predictive exploratory PICRUSt tool used in our work (**Fig. 2.7**) has provided us with potentially very useful information on the genomic and metabolic features of the bacterial communities of soil, rhizosphere and endosphere. The fact that proteins supposedly related to the reduction of arsenate, transport of arsenite and TE efflux systems, including the zinc transport system, were more numerous in the rhizosphere than in the bulk soil, was not a totally unexpected result, considering the possible presence of a TE concentration gradient in the vicinity of the plant, and therefore a more prominent TE-microorganism-plant interaction in that space. The same was the case, although to a lesser extent, with the conceivable presence of proteins related with ABC-transport systems (**Fig. 2.7**), which could be related with siderophore activities and the uptake of other essential nutrients in both, soil and rhizosphere, and/or with tolerance to harmful compounds (Naka et al., 2013; Du et al., 2015). A more balanced representation in soil and rhizosphere was observed for proteins related to the GSH metabolism, potentially related with defense against ROS and as a substrate for TE-chelating compounds (Fahey, 2013). On the other hand, the obvious presence of aquaporin proteins (**Fig. 2.7**) (mainly present in gram negative bacteria; Tanghe et al., 2006) could be related to osmoregulation and / or bacterial tolerance against adverse environmental conditions, as freezing, or even with a positive effect on the plant roots water status, through drought alleviation and salinity damage (Groppa et al., 2012). Aquaporins are also used by bacteria as an As extrusion mechanism that, as proved by the high As resistance demonstrated by the phenotypical characterization, could participate in this process. Presence of As-resistant bacteria (**Table 2.4**) in As/TE-rich environments was highly expected since high levels of TEs are likely to exert a strong selective pressure thereby reducing the overall growth of sensitive microbial communities (Sarkar et al., 2013). An interesting feature, which could directly influence the plant development was the

occurrence in the endophytic community, in substantial amounts relative to the soil and rhizosphere environments, of proteins hypothetically related with N fixation (**Fig. 2.7)**, which is in accordance with the phenotypical characterization of cultivable strains (**Table 2.4**). Finally, the high number of hypothetical flagellar proteins in the bulk soil compared to the rhizosphere suggests a lower proportion of mobile bacteria in the latter. This could be explained by the fact that, in order to avoid a plant systemic resistance response, phytopathogens and also endophytes, once attached to cell plants, lose their flagella or shield lipopolysaccharides to prevent their recognition by the plant cells (Gozzo, 2003).

When looking at the possible functions of bacterial microbiomes it is important to take into account that, as has been proposed by Berg et al. (2005), bacterial endophytes represent specialized members of bacterial groups that can also be indistinctly present in the rhizosphere. This can be illustrated by *Pantoea* sp., more abundant in bulk soil, although isolated from the endosphere, and *R. erythropolis*, more abundant in the endosphere, although isolated from the rhizosphere (**Fig. 2.4**). In any case, both strains harbor a remarkable array of PGP traits (**Table 2.4**) and therefore it can be expected *a priori* that they provoke changes in the growth and physiology of the plants, which might improve the TE tolerance and the phytoextraction efficiency (Conrath et al., 2006; Kong and Glick, 2017b; Ramakrishna et al., 2019).

2.5 CONCLUSION

The contribution of the plant-associated microbial populations (rhizosphere and even endosphere) is crucial in phytoremediation and cannot be neglected. Thus, the knowledge of plant-microorganism interactions is of great practical value to improve the efficiency of phytoextraction.

Microorganism-assisted phytoremediation is a very promising approach, that requires a previous exploration of the plant-associated microbial diversity, its distribution and function, and subsequently the selection and careful characterization of the most appropriate bacteria. The results of the sequence analysis of the total bacteria communities in our work showed differences in the composition of bacterial taxa in bulk soil and plant rhizosphere; for the endophytic fraction these results clearly suggest that root-microbes-plant interaction selects for specific bacterial taxa and thus exert some control shapes the microbiomes structure.

Total bacterial diversity decreases from bulk soil to endosphere. Five bacterial phyla were overrepresented in the willow microbiome: Proteobacteria (41.4%), Actinobacteria

(21.1%), Firmicutes (13%), Candidate division TM7 (9.4%) and Bacteroidetes (8.9%). Bacteria identified are related to genera *Pseudomonas* (17.9%), *Rhodococcus* (8.7%), *Pantoea* (8.7%), *Flavobacterium* (4.9%), *Paenibacillus* (4.3%), *Arthrobacter* (3.9%) and *Variovorax* (2.6%). The root endosphere was enriched with *Bosea*, *Bacillus*, *Rhizobium*, *Paenibacillus* and specially *Rhodococcus*. Genus *Pantoea* predominates in the bulk soil and *Pseudomonas* in the rhizosphere. A total of 136 bacterial strains associated with *S. atrocinerea* (65 endophytic and 71 rhizospheric) were isolated as axenic cultures. These bacteria distributed into three predominant phyla: Proteobacteria, Firmicutes, and Actinobacteria. Twenty-seven and eighteen different genera were identified from the endosphere and rhizosphere, respectively. *Bacillus* (30%) and *Pseudomonas* (14%) predominated in endophytes while *Bacillus* (27%) and *Streptomyces* (12%) were the most abundant in the rhizosphere.

An interesting feature, which could directly influence the plant development was the occurrence in the endophytic community, in substantial amounts relative to the soil and rhizosphere environments, of proteins hypothetically related with N fixation, which is in accordance with the phenotypical characterization of cultivable strains.

Chapter 3

Integrative response of Arsenic Uptake, Speciation and Detoxification by *Salix atrocinerea*

3 Integrative response of Arsenic Uptake, Speciation and Detoxification by *S. atrocinerea*

3.1 INTRODUCTION

Arsenic (As) is a metalloid widely spread in the upper Earth's crust although at very low concentrations. The overall mean value of the total As for different soils is estimated as 6.83 mg kg^{-1} soil. However, As soil concentrations may range from 0.1 to more than 1,000 mg kg^{-1} in some locations due to both anthropological and geological factors (Kabata-Pendias, 2010). Concerning its toxicity, As is the only known human carcinogen for which there is adequate evidence of carcinogenic risk for both exposure routes, inhalation and ingestion (Smith et al., 2009). Therefore, As has been defined as a group 1 carcinogen and is placed in the highest health hazard category by the international agency for research on cancer (Naidu et al., 2006). By the use of natural resources, humans release As into the air, water and soil (Mandal and Suzuki, 2002). Sixty percent of the anthropogenic As emissions can be accounted to only two sources: Cu-smelting and coal combustion. Nevertheless, the application of herbicides, Pb and Zn smelting, glass production, wood preservation, waste incineration and steel production are also responsible for As emissions (Matschullat, 2000). According to the European Commission (2000), air contributes less than 1% of the total As exposure since most of this emitted As ends up retained in the water and soils, making these the major sources of As exposure to humans.

Once inside the cell, As toxicity depends on its speciation state. Arsenite (As III) has a high affinity for sulfhydryl groups found in the amino acid cysteine. As such, it inactivates a wide range of enzymes by disrupting protein structure and impairs the metabolism by preventing protein–protein interactions (Ehlrich, 1990). This affects many key metabolic processes in the cell such as fatty acid metabolism, glucose uptake and glutathione production (Paul et al., 2007; Ahsan et al., 2008; Wang et al., 2015). Arsenate (As V) is a phosphate analogue and can substitute inorganic phosphate affecting ATP synthesis and therefore interrupting the production of energy, carbon metabolism and nucleic acid synthesis (Singh et al., 2011; Spratlen et al., 2017). This can also negatively affect DNA repair and methylation and thus impact on gene expression (Reichard and Puga, 2010). Therefore, removal or lowering of As concentrations from highly As-polluted soil and water is an environmental priority. Among the most eco-friendly cleanup technologies and opposite to traditional excavation and disposal in landfills, phytoremediation emerges. This green technology, already described more than two decades ago by Raskin et al. (1994), exploits the ability of certain plants species to accumulate metal(loid)s in their tissues, thus reducing

their concentrations or attenuating their mobility in the environment, and therefore offering a solution to the above-mentioned pollution challenge (Pilon-Smits, 2005; Kidd et al., 2015).

It is well known that toxic metal(loid)s induce loss of plant biomass, among other deleterious effects, mainly associated with growth inhibition (Gill et al., 2015). Plants differ in As tolerance, from sensitive plant species like all major crops, to tolerant plants such as certain ecotypes of the grass *Holcus lanatus* (Quaghebeur and Rengel, 2003), as well as hyperaccumulators like *Pteris vittata* (Chinese break fern), which can accumulate 2% of its dry weight as As (Wang et al., 2002). However, hyperaccumulator species are usually limited by a low biomass production, which may pose serious restrictions to this cleaning procedure (Shelmerdine et al., 2009, Fernández et al., 2010). Some plant species and soil biota populations, usually autochthonous to polluted soils, are able to colonize and thrive in highly polluted environments, even when high concentrations of metals are found in their cells and tissues. This is the case of *Salix atrocinerea* (grey willow). So far, about 450 species of *Salix* worldwide have been described (Argus, 1995), with some of them reported as suitable in phytoremediation processes because of their high growth rate and deep-rooting traits (Kuzovkina and Quigley, 2005; Janssen et al., 2015). Nevertheless, the focus on the use of *Salix* for As uptake is still low because it is not a metal(loid) hyperaccumulating species. However, some investigations have highlighted its phytoremediation potential for As (Purdy and Smart, 2008; Puckett et al., 2012; Yanitch et al., 2017). In addition, complementary studies exploring the feasibility of high biomass plants to extract metals from polluted soils such as willow, concluded that the high biomass compensates for the moderate metal concentrations found in the aboveground tissues (Hammer et al., 2003; Ruttens et al., 2011).

Understanding As tolerance in plants is useful to know whether plants avoid As uptake and, thus, reduce the As intake by humans and the As-associated health problems (Song et al., 2010), or enhance As uptake and its removal by phytoremediation (Yang et al., 2012). To achieve this, it is necessary to study the As behavior from the soil to its accumulation in the aboveground plant tissues. Although As is toxic and not essential for plants it is effectively absorbed through various transporters into the roots, mainly as As V, the most thermodynamically stable and hence dominant species in aerobic environments (Quaghebeur and Rengel, 2003). As such, As transporters include the high affinity phosphate uptake systems for As V (Shin et al., 2004; Catarecha et al., 2007; LeBlanc et al., 2013), while As III uses the silicon transporters (Xu et al., 2015; Lindsay and Maathuis, 2016). Once inside the plant cells, a small amount may be transported to the xylem but the majority is reduced to As III by arsenate reductases (Ellis et al., 2006; Duan et al., 2007; Zhao

et al., 2009). In this form, As can be exported back into soil, transported via the xylem to stem and leaves, or complexed with thiol-rich molecules like metallothioneins (MTs), glutathione (GSH) or, more stably, by phytochelatins (PCs) (Schmöger et al., 2000; Hartley-Whitaker et al., 2001a; Dave et al., 2013; Batista et al., 2014). Then these As-PCs complexes can subsequently be transferred from the cytosol into the vacuole by ABC transporters for storage in order to prevent cell damage (Song et al., 2010). Therefore, this suggests that non-protein thiols (NPTs) compounds play an important role in decreasing As toxicity in plants and preventing its transport from roots to shoots.

Apart from the works on arsenic with *Salix* of Purdy and Smart (2008), Puckett et al. (2012), and more recently the extensive transcriptomic study by Yanitch et al. (2017) that have provided unequivocal useful information to understand the tolerance of *Salix* to As, still an integrative approach concerning the tolerance mechanisms of *Salix* to As is needed. Besides, special attention needs to be paid to the speciation state of As, since this determines its uptake and also its tolerance by the plant. In the current study, a *S. atrocinerea* clone, previously selected for its As accumulation (unpublished data), was grown hydroponically in the presence of As V. Samples were harvested at different time points to kinetically study As accumulation and its chemical speciation in roots and shoots. In addition, the production of NPTs as well as the expression of the main transcripts involved in the genetic response behind As tolerance were also measured. Therefore, this study aims to describe the As uptake and accumulation in *S. atrocinerea*, together with the changes in the mechanisms involved in As tolerance at different biological organization levels.

3.2 MATERIAL AND METHODS

3.2.1 *Plant material and hydroponic culture conditions*

Salix atrocinerea plants were selected from an *in vitro* willow clone previously obtained from seeds collected at Nitrastur brownfield (Asturias, Spain). Stem cuttings of 15 cm length were placed on cellulose plugs in a hydroponic system containing 50 mL of 1/10 Woody Plant Medium (pH 5.7) (Lloyd, 1981) with an aeration system to prevent lack of oxygen (Moreno-Jimenez et al., 2010). After 3 weeks of growth, 48 cuttings were exposed to 0 and 18 mg L^{-1} As. This As concentration was similar to that found at the exchangeable fraction of the Nitrastur brownfield soil. The As was added as sodium heptahydrate arsenate, $Na_2HAsO_4.7H_2O$, since in this form the As is freely soluble.

Plants were cultured under a 12 h light photoperiod and 22 °C/18 °C with 65% relative humidity. Light was provided by a combination of blue, red and far-red Philips

Green-Power LED modules, simulating the photosynthetically active radiation (PAR) of sunlight. The PAR level reached 170 µmol m^{-2} s^{-1} at the plant apex level.

After 1, 3, 10 and 30 days (d), plants were carefully removed from beakers and roots exhaustively rinsed with tap water first, and 3 times with double de-ionized water at 4 °C. Leaves were rinsed only once in distilled water. To determine the influence of the treatments on plant growth, fresh and dry weights and lengths of roots and leaves were measured. Leaves and root samples of at least 3 different plants were analyzed individually for each treatment. Plant material was homogenized with liquid nitrogen and stored at −80 °C until further use.

3.2.2 Analysis of essential elements, arsenic and arsenic speciation

Nutrients, such as boron (B), calcium (Ca), iron (Fe), phosphorus (P), and zinc (Zn), together with As, were determined in leaves and roots of *S. atrocinerea*. For this, 100 mg of dry powdered samples were dissolved in 8 mL of 50% nitric acid solution (Sigma, Aldrich, USA) using a microwave at 800 W during 15 min (Multiwave3000, Anton Paar). The solutions were diluted up to 50 mL with ultrapure water and filtered through a 0.45 µm polytetrafluorethylene (PTFE) filter prior to their analysis. Plant samples were analyzed by ICP-MS (Agilent Technologies 7700 ICP-MS) using isotopic dilution analysis (IDA) as previously described (Gallego et al., 2015).

To determine the As speciation in leaves and roots, 100 mg of dry powdered samples were extracted in 2.5 mL of 0.3 M nitric acid solution at 95 °C for 90 min (Huang et al., 2012a). The extracts were centrifuged at 3000 *g* during 15 min and the supernatants were filtered through a 0.45 µm PTFE membrane filter. The solutions were neutralized by the addition of NaOH. The As species were separated through a mobile phase of 0.2 M EDTA dissolved in 2 M PBS (Phosphate Buffered Saline; pH 6.0) in a separation column with a 1260 Infinity HPLC coupled to a 7700 ICPMS (both from Agilent Technologies). Identification of As species was confirmed by spiking real extracts with a mixture of standard solutions: As III, As V, monomethylarsenic acid (MMA), and dimethylarsenic acid (DMA).

3.2.3 Analysis of non-protein thiolic compounds

The extraction and analysis of non-protein thiols (NPTs) were carried out from 150 mg of fresh weight leaves and roots of *S. atrocinerea* following the protocol described by Fernández et al. (2012). The high-performance liquid chromatography

(HPLC) separation was performed using a chromatograph Waters 600 (Waters Corporation) with a post-column derivatization with Ellman's reagent (Ellman, 1959). The sample (100 μL) was injected into a Kromasil 100 C18 5 μm (250 × 4.6 mm) column (Scharlau) and eluted with solvent A (acetonitrile: H$_2$O, 2: 98 (v/v) to which 0.05% trifluoroacetic acid (TFA) was added) and solvent B (acetonitrile: H$_2$O, 98: 2 (v/v) also with 0.05% TFA). Samples were separated using a linear gradient (0–25% in 25 min and 25–50% in 5 min) of solvent B at 1.5 mL min^{-1} flow for 30 min. The derivatized thiols were detected at 412 nm using a Waters 996 photodiode array detector and the obtained peaks were identified by comparison with the standards of GSH and a mix of PCs. The quantitative changes in the thiol compounds observed were calculated by the integration of their peak areas at 412 nm converted into nmol and quantified as GSH equivalents.

3.2.4   *Gene expression analysis*

Isolation of RNA was carried out using the protocol described by Chang et al. (1993) with slight modifications. Frozen leaves or roots (100 mg) were homogenized with 550 μL of buffer containing 2% hexadecyltrimethylammonium bromide (CTAB), 2% polyvinylpyrrolidone (PVP), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.5 g L^{-1} spermidine and 2% β-mercaptoethanol. Then, it was extracted twice by adding 550 μL of chloroform:isoamyl alcohol (24:1) and centrifuged at 14,000 *g* for 20 min at 4 °C. After addition of 10 μL LiCl (10 M), RNA was precipitated overnight at 4 °C and harvested by centrifugation at 14,000 *g* for 20 min at 4 °C. The pellet obtained was washed with 75% ethanol and resuspended in RNase free water. The concentration of RNA was determined spectrophotometrically at 260 nm using Nanodrop equipment (Isogen Life Science) and the RNA quality was tested using the Experion™ automated electrophoresis system (Bio-Rad). DNA was removed using a TURBO DNA-free Kit (Ambion) and the cDNA synthesis was performed using PrimerScript RT reagent Kit (Takara) with equal amounts of RNA input (1 μg). Finally, the cDNA was ten-fold diluted using a 1/10 dilution of TE (Tris-EDTA) buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at −20 °C.

Reverse Transcription quantitative PCR (RT-qPCR) was performed with an ABI Prism 7900HT Fast Real Time PCR system (Applied Biosystems), using Fast SYBR Green chemistry. Gene forward and reverse primers (**Supplementary Table 1**) were designed using Primer 3 (Untergasser et al., 2012), according to sequences of genes obtained in the *Phytozome* nucleotide database of the closely related species *Salix purpurea* v1.0, for which

the whole genome has been sequenced (Goodstein et al., 2012), only for three genes, high-affinity phosphate transporter (*HAPO4*), arsenite-inducible RNA-associated protein (*AIP-1*) and metallothioneins (*MT1A*), their sequences were obtained from willow reference sequences annotated at the NCBI (National Center for Biotechnology Information) (O'Leary et al., 2016). Their specificity was verified *in silico* using BLAST (http://www.arabidopsis.org/Blast/index.jsp). The genes measured (**Supplementary Table 1**) were selected based on different genetic aspects behind As tolerance (Konlechner et al., 2013; Puckett et al., 2012; Yanitch et al., 2017). The qPCR efficiency of the primers was determined using a standard curve consisting of a two-fold dilution series of a pooled sample. Only primers with an efficiency between 90 and 110% were used for qPCR analysis and their amplification specificity was validated by melting curves. PCR amplifications were done in a total volume of 10 μL containing 2 μL cDNA sample, 5 μL SYBR Green, 0.6 μL of primers (300 nM) and 2.4 μL RNase free water. The reaction cycle was as follows: 20 s at 95 °C, 40 cycles of 1 s at 95 °C and 20 s at 60 °C. Gene expression was calculated relatively as $2^{-\Delta Cq}$, in which ΔCq represents each corresponding quantification cycle (Cq) value minus the minimum Cq value observed (Schmittgen, 2008). Gene expression was normalized with a normalization factor based on the expression of six reference genes from *Salix* selected from literature under As and other abiotic stresses (Li et al., 2016; Zhang et al., 2017). The 6 selected candidate reference genes, *α-TUB2, Alpha-tubulin 2; ACT7, Actin 7; ARF2, ADP-ribosylation factor 2; DNAJ, Chaperone protein DnaJ 49; EF1α, Elongation factor 1-alpha* and *OTU; OTU-like cysteine protease* (**Supplementary Table 1**) are also orthologs of genes in *S. purpurea*. The primer sequences, amplicon length, PCR amplification efficiency and correlation coefficient are shown in **Supplementary Table 1**. To evaluate the stability of the 6 candidate reference genes (RG) at the transcript level under As exposure, the gene expression levels were determined by the average Cq values. In order to detect the stabilities of 6 candidate RGs, the best combination of RG for normalization of our transcripts of interest was suggested by the Graynorm algorithm (Remans et al., 2014). In roots *AFR2*, *OTU* and *EF1α* were the three most stable reference genes in all the sample sets according to the *GrayNorm* algorithm and the combination of the three was used for normalization. In leaves a different combination of genes than that obtained in roots showed the most stable pattern in all the sample sets, and therefore a combination of *α-TUB2*, *OTU* and *ACT7* was used for normalization. In both roots and leaves the suggestion selected by *GrayNorm* corresponded to the genes less affected by As exposure.

A principal component analysis and heat maps were constructed to compare expression levels between different genes and samples at different time points.

3.2.5 *Statistical analysis*

To evaluate the effects of As toxicity in *S. atrocinerea* over the different time points on the measured variables, depending on the number of variables to compare a one-way or a two-way Analysis of variance (ANOVA) was performed. Log transformation was applied to approximate when it was necessary (e.g. to determine statistical significance of gene expression data, datasets were first log-transformed). Data normality was tested using the Shapiro-Wilk test, while homoscedasticity was verified via Bartlett's and Levene's tests. If data did not meet the normality assumption, a non-parametric Kruskall-Wallis test was used, followed by the Wilcoxon rank sum test. When the F ratio was significant ($p \leq 0.05$), Tukey's least significant difference test (HSD, $p \leq 0.05$) was employed to compare between individual means of different data groups (e.g. different treatments). In the gene analysis the previous was performed on both the normalized and the non-normalized data, although only the first are presented both were taking into account to establish the significance of the results. Results are expressed as the mean ± standard deviation of at least three independent replicates. All data were analyzed using R (version 3.3.1, http://www.r-project.org/) with the packages mixOmics (for PCA, version 6.0.1, http://www.mixOmics.org) and agricolae (version 1.2e4, http://tarwi.lamolina.edu.pe/~fmendiburu). Outliers were determined using the Extreme Studentized Deviate method (GraphPad Software, La Jolla, CA, USA) at significance level $p \leq 0.05$.

3.3 RESULTS

3.3.1 *Plant growth and nutrient analysis*

After 30 d of exposure, no external symptoms of phytotoxicity (data not shown) nor growth reduction measured as dry weight were observed between plants grown on control or As-containing medium (**Fig. 3.1**).
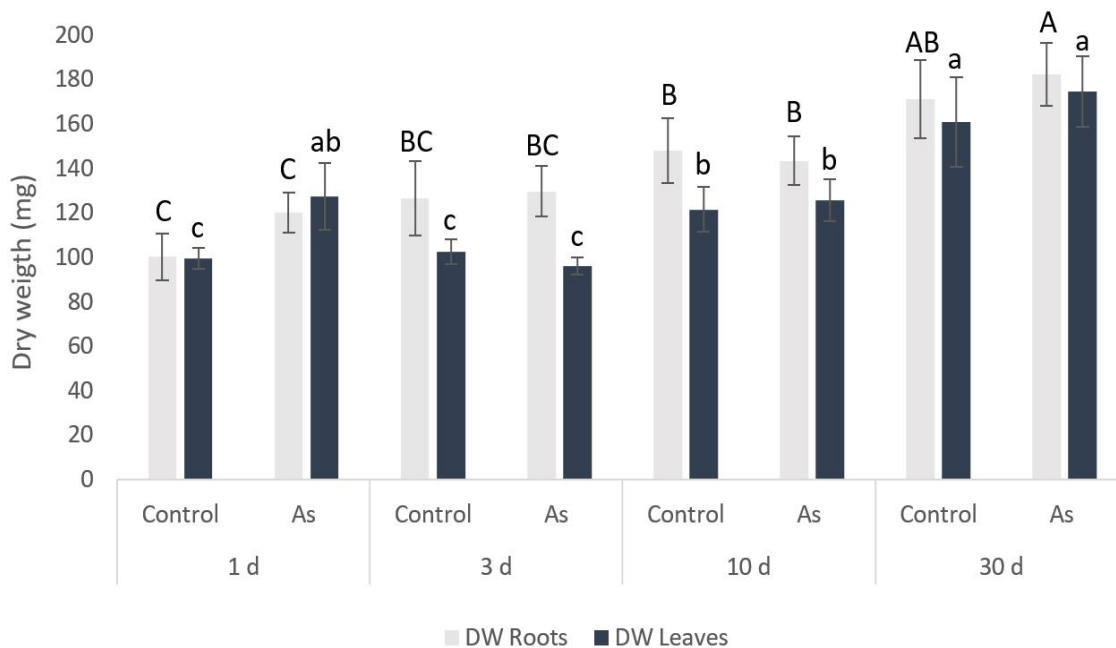
Fig. 3.1. Dry weight (DW) of roots and leaves of *S. atrocinerea* exposed to control and arsenic conditions for 30 days. Different letters (upper case for comparison within roots and lower case for comparisons within leaves) denote significant differences on HSD test at p < 0.05.

With regard to nutrient concentrations, total P concentration significantly decreased in roots from 10 d onwards in As-exposed plants as compared to controls, whereas in leaves the P concentration was lower in As-exposed plants as compared to controls at 3 and 10 d. However, P concentration in leaves was similar in both treatments after 30 d of exposure (**Table 3.1**). Accumulation of Ca increased in As-exposed roots along the exposure time when compared to control conditions. However, in leaves a Ca decrease was observed in As-exposed plants, except at 3 d (**Table 3.1**). Although the B concentration was slightly higher in roots of As-exposed plants, this increase was only significant at 3 d. In leaves, an increase in B concentration at 3 d was observed and this increase was maintained in As-exposed plants till the end of the experiment. For Zn concentrations, there was an increase in roots and a decrease in leaves as compared to control conditions at 1 d of As exposure. However, no differences were observed in for this elements at other time periods. Fe concentrations were higher in roots of As-exposed plants throughout the experiment, whereas the opposite trend was observed in leaves (**Table 3.1**).

Table 3.1. Nutrients (mg kg^{-1} DW) in roots and leaves of *S. atrocinerea* exposed to control and arsenic conditions for 30 days. Different letters within each column and tissue indicate significant differences among treatments and time points on HSD test at p < 0.05.

Organ	Time (d)	Treatment	Nutrient				
			P	Ca	B	Zn	Fe
Roots	1	Control	5757.89 ± 430.12 a	4322.53 ± 207.32 b	19.62 ± 1.12 a	512.35 ± 53.12 b	1955.93 ± 98.36 b
		As	5720.65 ± 379.34 a	4929.41 ± 242.98 a	21.98 ± 1.23 a	625.22 ± 29.75 a	2364.78 ± 115.93 a
	3	Control	5791.72 ± 456.56 a	3271.75 ± 245.56 b	15.83 ± 0.89 c	439.97 ± 23.45 bc	1219.43 ± 62.34 d
		As	5507.21 ± 412.42 a	4556.62 ± 342.45 a	19.46 ± 0.93 a	469.75 ± 30.45 bc	1354.59 ± 49.45 c
	10	Control	3772.06 ± 235.67 b	3041.54 ± 289.87 c	15.53 ± 0.92 b	481.45 ± 32.34 bc	1087.50 ± 83.12 e
		As	2883.34 ± 176.34 c	4257.52 ± 458.96 b	17.53 ± 1.09 ab	432.36 ± 23.56 c	1287.73 ± 69.32 c
	30	Control	2729.45 ± 278.45 d	3093.95 ± 334.56 c	13.26 ± 1.01 d	330.76 ± 22.34 d	943.27 ± 34.23 f
		As	1633.24 ± 99.83 e	4154.46 ± 354.98 a	14.38 ± 0.89 d	332.44 ± 15.69 d	1082.25± 50.54 e
Leaves	1	Control	3403.13 ± 179.33 a	6227.33 ± 434.93 a	64.22 ± 6.73 ab	633.62 ± 40.93 a	234.31 ± 12.45 b
		As	3112.77 ± 143.54 ab	5290.13 ± 302.34 b	49.23 ± 5.34 bc	544.83 ± 30.87 b	226.23 ± 10.15 b
	3	Control	3591.96 ± 123.43 a	5886.58 ± 478.23 b	49.31 ± 3.53 bc	646.95 ± 51.23 a	382.15 ± 21.54 a
		As	2942.52 ± 174.23 b	6909.65 ± 398.12 a	68.55 ± 4.52 a	532.08 ± 79.56 ab	180.67 ± 7.28 c
	10	Control	2358.36 ± 132.34 c	6378.05 ± 403.23 ab	47.97 ± 2.23 c	503.68 ± 23.13 b	250.83 ± 10.23 b
		As	2046.62 ± 124.54 d	4603.75 ± 345.21 c	57.26 ± 3.21 b	516.58 ± 31.22 b	143.65 ± 18.23 d
	30	Control	2035.46 ± 121.23 d	7001.72 ± 421.23 a	43.32 ± 2.34 d	429.35 ± 28.78 c	239.40 ± 12.12 b
		As	2106.37 ± 134.24 cd	5492.63 ± 324.12 b	60.67 ± 3.11 ab	400.92 ± 33.21 c	152.47 ± 13.52 d

With regard to the pH of the culture medium, we generally observed a decrease during the first 3 days of the experiment. However, an increase from 10 d onwards was observed under As exposure as compared to control medium (**Fig. 3.2A**).
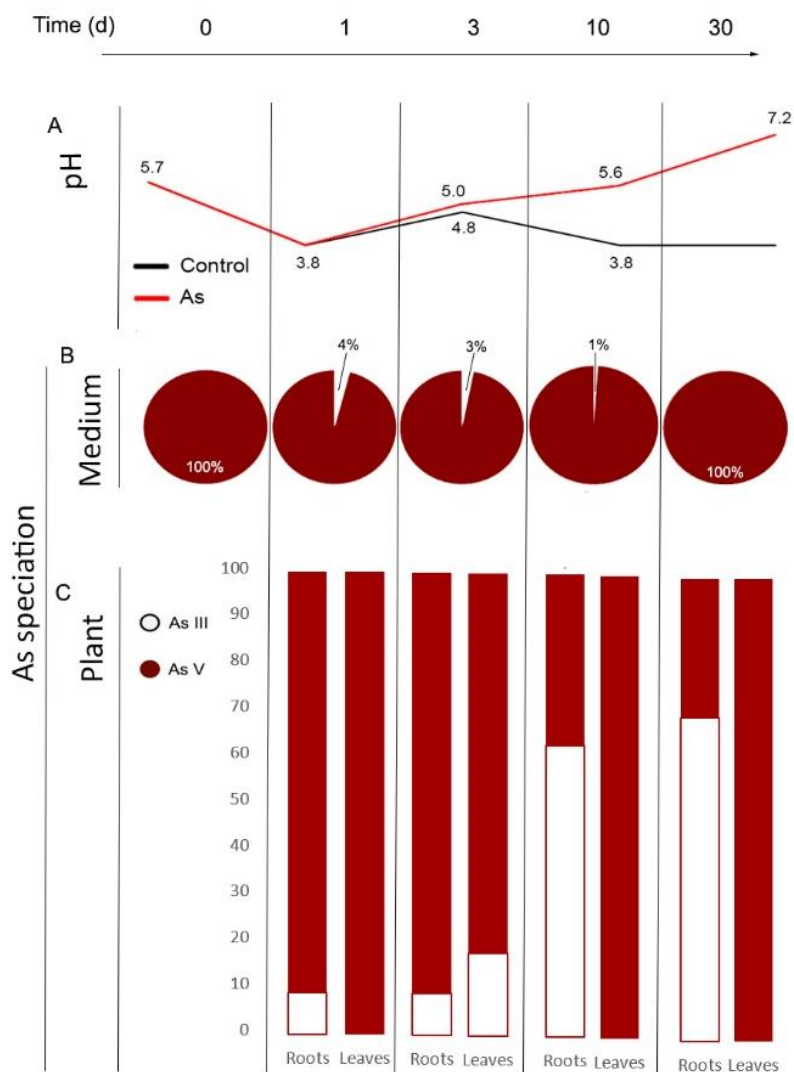
Fig. 3.2. pH in the culture medium **(A).** Percentage of arsenic speciation in the culture medium **(B)** and roots and leaves **(C)** of *S. atrocinerea* exposed to arsenic for 30 days (Red: As V, white: As III).

3.3.2   *Arsenic accumulation and speciation*

We observed that roots of *S. atrocinerea* accumulated As concentrations ranging from 180 mg As kg^{-1} dry weight at 1 d to more than 2,400 mg As kg^{-1} dry weight after 30 d of exposure (**Table 3.2**). In leaves, As accumulation was much lower, although after 30 d of

exposure, it reached an As concentration higher than that present in the culture medium (**Table 3.2**). Although only As V was added to the culture medium, 4% of As III was observed in the medium after 1 d of exposure and it decreased to 0% by the end of the experiment (**Fig. 3.2B**). Total As concentration in the medium decreased 14 % due to plant uptake and no spontaneous As speciation was detected in the medium when *S. atrocinerea* was not present (data not shown).

In plant tissues, the As was detected as As III or As V, but no As methylated species were observed (**Table 3.2**). In roots, As V was more abundant (91%) during the first 3 d of exposure but after 10 d, As III was the predominant As form (**Fig. 3.2C**). In leaves, As V was the predominant speciation form observed throughout the experiment and As III was only detected at 3 d of exposure in low quantity (18%) (**Fig. 3.2C**).

Table 3.2. Arsenic accumulation (mg kg^{-1} DW) in roots and leaves of *S. atrocinerea* exposed to arsenic for 30 days. Different letters within each column and plant tissue indicate significant differences among time points on HSD test at $p < 0.05$. nd: not detected.

Organ	Time point (d)	Arsenic		
		III	V	Total
Roots	1	16.14 ± 2.34 d	164.88 ± 149.33 d	182.43 ± 20.10 d
	3	33.45 ± 4.78 c	318.86 ± 21.95 c	353.65 ± 23.98 c
	10	929 ± 80.21 b	542.35 ± 41.29 b	1471.92 ± 123.87 b
	30	1688 ± 148.43 a	734.90 ± 65.20 a	2448 ± 178.32 a
Leaves	1	nd	2.78 ± 0.24 d	2.78 ± 0.24 d
	3	1.30 ± 0.08 e	5.76 ± 0.45 c	7.23 ± 0.39 c
	10	nd	18.75 ± 1.14 b	18.75 ± 1.14 b
	30	nd	25.45 ± 2.57 a	25.45 ± 2.57 a

In plant tissues, the As was detected as As III or As V, but no As methylated species were observed (**Table 3.2**). In roots, As V was more abundant (91%) during the first 3 d of exposure but after 10 d, As III was the predominant As form (**Fig. 3.2C**). In leaves, As V was the predominant speciation form observed throughout the experiment and As III was only detected at 3 d of exposure in low quantity (18%) (**Fig. 3.2C**).

3.3.3   *Analysis of non-protein thiolic compounds*

In roots of control plants only GSH was observed and present at a 2-fold higher concentration than that observed in the As-exposed roots (**Table 3.3**). Nevertheless, already after 1 d of exposure, changes in the concentrations of non-protein thiols (NPTs) in roots and leaves were observed in As-exposed plants and this trend was maintained over time (). Interestingly, in the roots of As-exposed plants the total concentration of NPTs increased over time (up to 4.5-fold higher after 30 d compared to 1 d) and it was always higher than that observed in leaves. This NPTs increase in roots under As exposure was mainly due to an increment of *de novo* synthesized compounds such as PC_2, $Cys\text{-}PC_2$, PC_3, $desGly\text{-}PC_3$, $Cys\text{-}PC_3$ and also two unidentified thiolic compounds that were named TC_1 and TC_2 (**Table 3.3**).

In leaves of control plants, the thiolic compounds GSH, $desGly\text{-}PC_4$, and TC_3 were detected (**Table 3**), whereas under arsenic exposure we observed *de novo* synthesis of $desGly\text{-}PC_2$ at increasing concentrations over time. In both control and As-exposed plants, GSH concentrations in leaves were always higher than those observed in roots and were initially higher in As-exposed plants than in control plants (**Table 3.3**). This increase in GSH, together with *de novo* synthesis of $desGly\text{-}PC_2$, accounted for a higher NPTs concentration at 1 d and 3 d in leaves of As-exposed plants. However, after 10 and 30 d of exposure, the total NPTs concentration in leaves of plants exposed to As did not significantly differ from that observed in leaves of plants grown under control conditions (**Table 3.3**).

Table 3.3. Non-protein thiolic peptides (nmol GSH g^{-1} FW) in roots and leaves of *S. atrocinerea* exposed to control and arsenic conditions for 30 days. Different letters within each row and plant tissue indicate significant differences among treatments and time points on HSD test at $p < 0.05$. nd: not detected.

Organ	Thiol	1 d Control	As	3 d Control	As	10 d Control	As	30 d Control	As
Roots	Cys	nd	5.85 ± 1.73 a	nd	19.38 ± 0.18 b	nd	7.99 ± 2.15 a	nd	4.06 ± 0.15 c
	GSH	13.73 ± 0.93 a	7.90 ± 0.66 b	11.68 ± 1.59 a	6.91 ± 0.55 bc	15.15 ± 1.83 a	6.15 ± 0.74 c	12.80 ± 1.40 a	6.05 ± 0.52 c
	TC_1	nd	14.69 ± 1.11 a	nd	10.62 ± 0.74 b	nd	6.72 ± 1.25 c	nd	nd
	PC_2	nd	13.63 ± 1.85 b	nd	16.72 ± 1.78 ab	nd	19.10 ± 1.03 a	nd	18.42 ± 1.75 a
	$Cys-PC_2$	nd	10.37 ± 0.76 d	nd	13.46 ± 1.51 c	nd	23.13 ± 0.02 b	nd	34.35 ± 1.48 a
	TC_2	nd	6.78 ± 0.14 d	nd	10.17 ± 0.32 c	nd	17.12 ± 0.45 b	nd	21.79 ± 0.29 a
	PC_3	nd	20.32 ± 1.40 d	nd	33.64 ± 1.09 c	nd	47.01 ± 9.54 b	nd	65.38 ± 1.06 a
	$desGly-PC_3$	nd	8.88 ± 0.53 d	nd	34.35 ± 2.27 c	nd	73.86 ± 4.27 b	nd	150.19 ± 12.24 a
	$Cys-PC_3$	nd	10.91 ± 0.71 d	nd	61.14 ± 1.74 c	nd	74.34 ± 3.73 b	nd	169.27 ± 11.71 a
	Total ΣNPTs	13.73 ± 0.93 e	99.34 ± 2.71 d	11.68 ± 1.59 e	174.85 ± 18.70 c	15.15 ± 1.83 e	267.57 ± 12.93 b	12.80 ± 1.40 e	469.52 ± 21.32 a
Leaves	GSH	43.09 ± 2.53 b	49.35 ± 1.80 a	40.30 ± 3.45 bc	49.87 ± 3.33 a	44.70 ± 2.78 ab	37.74 ± 1.88 c	45.63 ± 2.45 ab	41.41 ± 1.86 b
	$desGly-PC_2$	nd	2.22 ± 0.31 d	nd	5.18 ± 1.03 c	nd	7.65 ± 0.53 b	nd	9.39 ± 0.88 a
	$desGly-PC_4$	2.50 ± 0.11 d	2.52 ± 0.10 d	2.46 ± 0.14 d	3.06 ± 0.36 c	2.74 ±0.12 cd	2.87 ± 0.14 c	4.09 ± 0.52 b	6.46 ± 0.48 a
	TC_3	4.48 ± 0.38 b	5.45 ± 0.23 a	4.04 ± 0.49 b	6.25 ± 0.64 a	4.04 ± 0.34 b	5.08 ± 0.70 ab	3.03 ± 0.18 c	3.95 ± 0.41 b
	Total ΣNPTs	55.40 ± 1.89 bc	67.60 ± 1.45 a	50.54 ± 3.45 c	69.58 ± 5.70 a	53.56 ± 4.05 bc	58.16 ± 2.50 b	54.70 ± 4.11 bc	62.51 ± 5.94 ab

3.3.4 Gene expression

In general, the gene expression pattern in roots between control and As-treated samples differed due to the prominent regulation of transcripts related to As transport, As V reduction to As III, thiol metabolism and vacuolar transports. In this way changes were observed in transcripts coding for the phosphate transporter (PHO1), aquaporins (NIP1, SIP1 and SILICON), boron transporter (BORON), As V reductase CDC25-like tyrosine phosphatase (CDC25-1), glutathione synthase (GS) and ABC transporter (WBABCT) (**Fig. 3.3**).
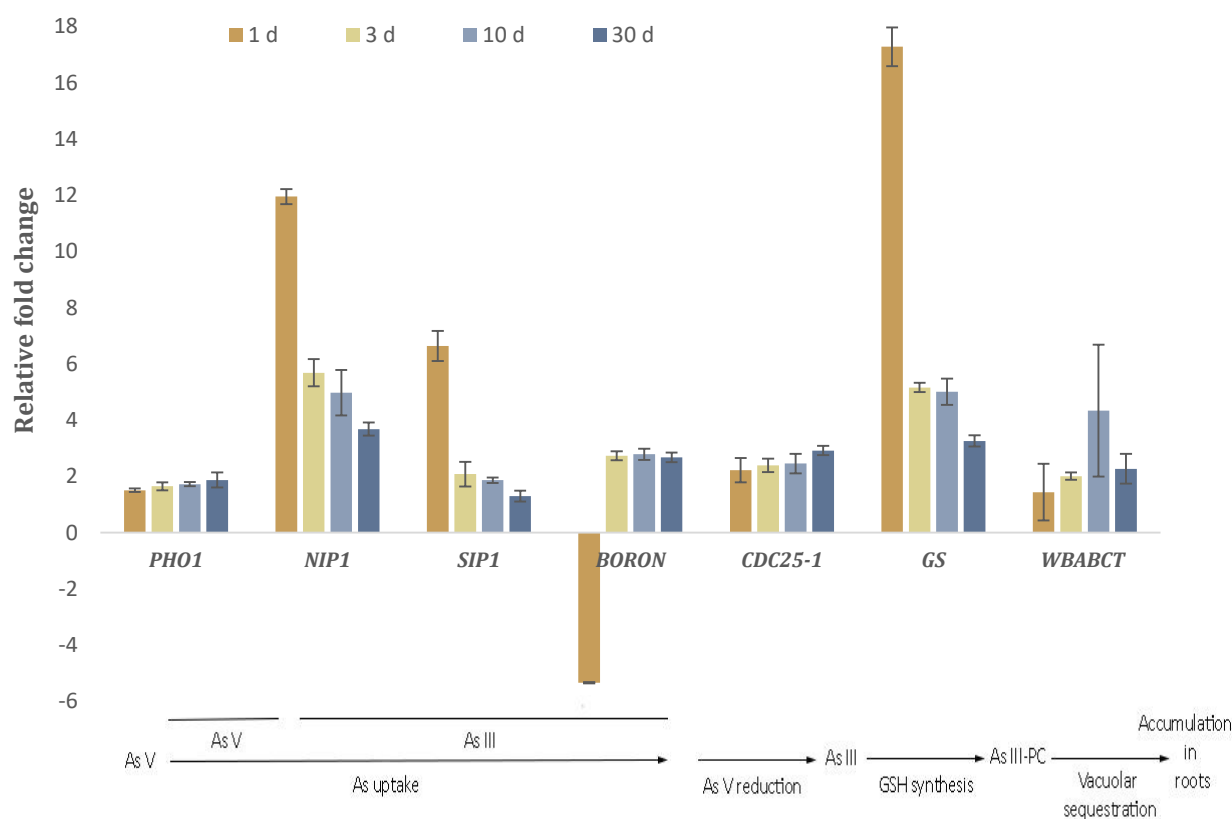
Fig. 3.3. Relative fold change of the gene expression levels in roots of *S. atrocinerea* exposed to As regarding those genes involved in As uptake and reduction, thiol synthesis and vacuolar sequestration, that showed the most markedly regulation along the 30 days. Values represented are the fold change (± S.D.) of mean normalized expression relative to the non-exposed plants at each time point of at least three biological replicates, each containing at least one individual plant. *BORON*, boron transporter*; CDC25-1*, tyrosine phosphatase 1, *GS*, glutathione synthetase; *NIP1*, aquaporin NIP1.1; *PCS*, phytochelatin synthase; *PHO1*, phosphate transporter PHO1; *SIP1*, aquaporin SIP.1; *WBABCT*, white-brown-complex ABC transporter.

This differential regulation was also accompanied by changes in transcripts for As stress-related proteins like cellulose synthase (*CSA*), arsenite inducible protein (*AIP-1*) and aminocyclopropane-1-carboxylate synthase (*ACCS*). In leaves, regulation of the transcripts measured was not so noticeable as in roots and differences in gene expression between control and As-exposed plant were due mainly to the overexpression of ACCS (**Fig. 3.4**).
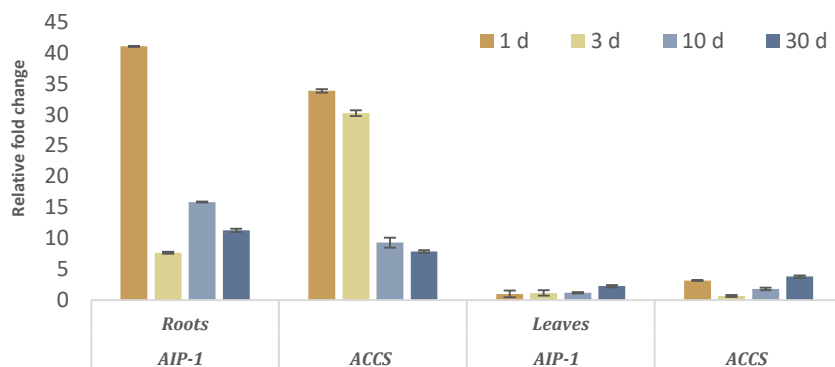
Fig. 3.4. Relative fold change of the gene expression levels in roots of *S. atrocinerea* exposed to As regarding those genes involved in stress response that showed the most markedly regulation along the 30 days. Values represented are the fold change (± S.D.) of mean normalized expression relative to the non-exposed plants at each time point of at least three biological replicates, each containing at least one individual plant. *ACCS*, aminocyclopropane-1-carboxylate synthase; *AIP-1*, arsenite-inducible RNA-associated protein AIP-1-related.

A heat map representation of the other transcripts measured in this study with a fold regulation lower than two can be found for both roots (**Fig. 3.5A**) and leaves (**Fig. 3.5B**).
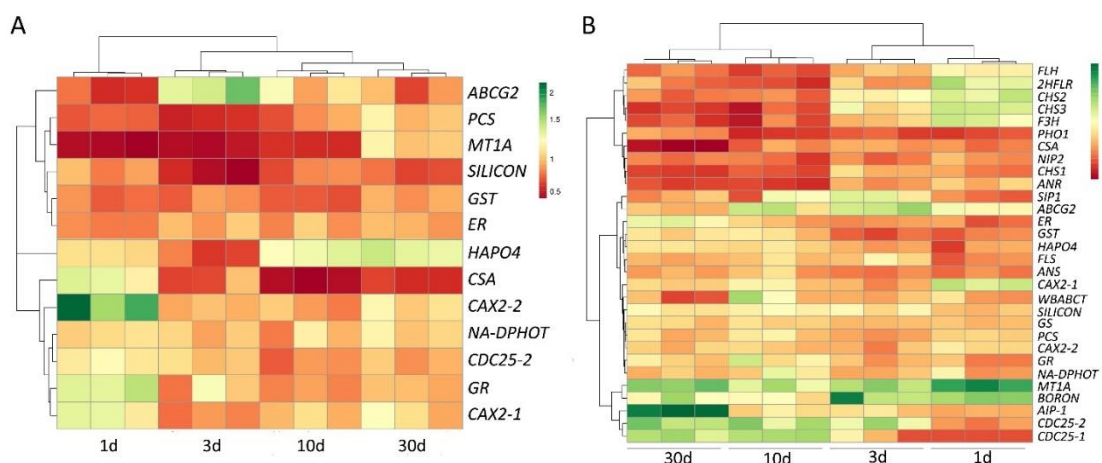
Fig. 3.5. Heat map representations of the gene expression data obtained in samples of roots **(A)** and leaves **(B)** of *S. atrocinerea* exposed to arsenic for 30 days and hierarchical clustering based on the most differentially expressed genes with a fold regulation equal or lower than 2. Gene expression level values are the normalized expression relative to the non-exposed plants at each time point of at least three biological replicates, each containing at least one individual plant. Green-shaded rectangles indicate increased, while red-shaded rectangles indicate decreased gene expression. For gene abbreviations see *Supplementary Table 1.*

To establish the kinetic gene expression of related target genes, a principal component analysis (PCA) was performed using the gene expression data obtained in leaves and roots of *S. atrocinerea* plants collected at 1, 3, 10 and 30 d. According to PCA component 1, roots of plants exposed to As for 1 d showed the highest gene expression for *GS, NIP1, SIP1, CSA, AIP-1,* vacuolar transporter (*CAX2-2*), glutathione reductase (*GR*) and *ACCS*, whereas the expression of *BORON*, *PHO1*, *MT1A, CDC25-2, WBABCT*, vacuolar transporter (*ABCG*) was higher at later time points. On the other hand, PCA component 2 in roots (24% of the total variation) indicated that the increased expression of *ABCG*, clustered samples at 3 d, whereas the decrease in expression for phytochelatin synthase (*PCS*) and transcripts for a high-affinity phosphate transporter (*HAPO4*) at 3 d separated this group from the rest (**Fig. 3.6A**).
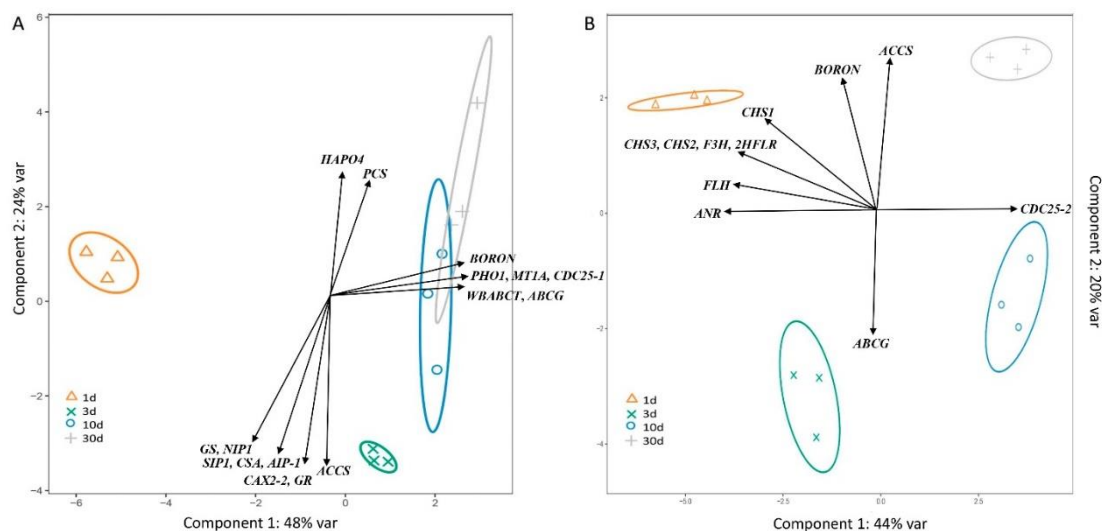
Fig. 3.6. Biplots of the principal component analysis (PCA) in samples of roots **(A)** and leaves **(B)** of *S. atrocinerea* exposed to arsenic for 30 days calculated with the normalized gene expression levels relative to the non-exposed plants at each time point of at least three biological replicates, each containing at least one individual plant. *2HFLR*, dihydroflavonol 4-reductase; *ABCG*: ABC transporter G; *ACCS*, aminocyclopropane-1-carboxylate synthase; *AIP-1*, arsenite-inducible RNA-associated protein AIP-1-related; *ANR*, anthocyanidin reductase; *BORON*, boron transporter*; CAX2-2*, vacuolar cation/proton exchanger 2; *CDC25-1, 2*, tyrosine phosphatase 1, 2; *CHS1,3*, chalcone synthase 1,3; *CSA*, cellulose synthase A; *F3H*, flavanone 3-hydroxylase; *FLH*, Flavonoid 3'-hydroxylase; *GR*, glutathione reductase; *GS*, glutathione synthetase; *HAPO4*, high-affinity phosphate transporter 4; *MT1A*, Metallothionein; *NIP1*, aquaporin NIP1.1; *PCS*, phytochelatin synthase; *PHO1*, phosphate transporter PHO1; *SIP1*, aquaporin SIP.1; *WBABCT*, white-brown-complex ABC transporter.

In leaves, according to PCA component 1, the differential gene expression collected from plants growing under As exposure at 1 and 3 d, had a more similar pattern than that observed at 10 and 30 d. Main differences were attributed to the up-regulation of genes

involved in the flavonoid pathway *CHS3*, *CHS2*, *ANR*, *F3H*, *FLH*, *2HFLR*, *BORON* and *CHS1* expression at 1 and 3 d and of *CDC25-2* at 10 and 30 d. Component 2 (20% of the total variation), however, indicated a separation between the initial (1 d) and the last time point (30 d) from the intermediate points (3 and 10 d) as a consequence of lower *ACCS* and higher *ABCG2* expression in these intermediate points (**Fig. 3.6B**).

3.4   DISCUSSION

The total pollutant concentration of a certain element in the soil is not a representation of the amount that is available (exchangeable) for the plant uptake, neither a good indicator to establish plant toxicity limits. Therefore, when phytoremediation processes will rely on the use of certain plant species that tolerate and accumulate high concentrations of metal(loid)s, it is very important that the studies conducted in the laboratory under controlled conditions, on which the basic physiological knowledge is set, are based on well reflected pollutant concentrations. Many hydroponic studies have used higher As concentrations than those found in soil solution, and their environmental relevance has been questioned (Fitz and Wenzel, 2002). According to this, some authors propose that hydroponic cultures should include As doses in the range of 0 – 10 µM to allow the extrapolation of the results to As-polluted soils (Moreno-Jimenez et al., 2010). Nonetheless, the fact that *S. atrocinerea* plants used in this study already grow on a brownfield with an As exchangeable fraction of 18 mg kg^{-1} (data not shown), suggests that the As dose could be increased for this hydroponic assay. Furthermore, this As concentration matches that recommended in previous hydroponic studies with willow (Purdy and Smart, 2008) and it has already been used in analyzing differential As gene expression under hydroponic conditions (Puckett et al., 2012). Although some authors have reported that willows have the capability to translocate As from roots to aboveground tissues (Tlustoš et al., 2007; Puckett et al., 2012; Sylvain et al., 2016) the As accumulation in leaves does not reach those quantities present in hyperaccumulating species like *Pteris vittata* (Caille et al., 2004), as it was our case, but the phytoremediation potential is compensated with a higher biomass (Meers et al., 2007, Witters, 2009a). Furthermore, after 30 d of As exposure, *S. atrocinerea* did not show any phytotoxic symptoms and was capable of accumulating a higher As concentration than that present in the culture medium, showing therefore a bioaccumulation factor higher than 1, which is an added value for the phytoremediation of As.

It has been reported that exposure to toxic metalloids, such as As, can disturb the nutrient profile of the plant and hence lead to toxicity (Lou et al., 2010), and also that As V

uptake and tolerance to its induced toxicity is intimately linked to phosphate nutrition. In the soil, As is mainly present in its As V form (Cordos et al., 2006) and once it is in contact with the roots, As V can enter via phosphate transporters (Maciaszczyk-Dziubinska et al., 2012). Therefore, changes in transcripts encoding for As V-related transporter proteins could be expected and, in our case, the As V added to the hydroponic solution caused a differential regulation of transcripts for phosphate transporters. The up-regulation of *PHO1* in roots of *S. atrocinerea* from the onset of the As exposure (**Fig. 3.3**) and that observed at 10 and 30 d of transcripts encoding for a high-affinity phosphate transporter protein (*HAPO4*) (**Fig. 3.5A**), relate to the first lower and then similar P concentrations in roots of As-exposed *S. atrocinerea* as compared to non-exposed plants (**Table 3.1**). It has been suggested that reduced uptake of As V is a well-known mechanism of As V resistance employed by many plant species, which is achieved through a reduction of the phosphate/arsenate uptake system in resistant plants (Meharg and Hartley-Whitaker, 2002). Moreover, it is thought that this reduction decreases As V influx to a level at which the plant can detoxify As, presumably by constitutive mechanisms (Catarecha et al., 2007). However, according to our results of As accumulation and a lower concentration of P under As exposure as compared to the control condition, it can be suggested that the transcript upregulation of phosphate-related transcripts in roots is based on preventing As V competition and avoiding P deprivation. Therefore, since As does accumulate at high concentrations in roots of willow, a more effective detoxification mechanism than inhibition of phosphate transporters as seen in other plants would be necessary in *S. atrocinerea*. After 30 d, As concentration in leaves of *S. atrocinerea* reached levels higher than toxicity levels established for non-tolerant plants (1-20 mg As kg^{-1} dry weight; White and Brown, 2010). Under these conditions, a differential regulation of As V-related transporters in leaves of *S. atrocinerea* was observed. The decrease in *PHO1* transcripts at 1, 3 and 10 d (**Fig. 3.5B**), is a similar response to that of As resistant species, where avoiding As uptake in leaves by reducing phosphate uptake constitutes a tolerance mechanism (Meharg and Hartley-Whitaker, 2002). However, at 30 d the down-regulation ceased and there were no differences in transcript levels of *PHO1* compared to those observed in leaves of control plants and it matched with a similar P concentration in leaves of both treatments (**Table 3.1**). According to the Ca concentrations observed in plants of *S. atrocinerea* (**Table 3.1**), it can be suggested that Ca accumulation in the roots and its reduced translocation to the leaves is a response to As accumulation. Ca is an essential plant macronutrient and it plays an important role in cell wall and membrane stabilization and regulates nutrient uptake as well as different stress responses (Ahmad et al., 2015), including an increase of the antioxidant defense under As exposure and reducing As uptake (Rahman et al., 2015). In

multiple studies, it has been shown that micronutrient accumulation is affected by As exposure, but it can also have an impact on As uptake and hence As toxicity (Srivastava et al., 2017). It has been proposed that B channels might have a role in As transport into the cell (Yanitch et al., 2017), which is also reflected in our results with increased *BORON* transcript levels in roots (**Fig. 3.3**) and leaves (**Fig. 3.5B**). Furthermore, boric acid transporter NIP5.1 from *Arabidopsis* is also permeable to As III (Mitani-Ueno et al., 2011), and our data showed that B accumulation in plant tissues changes along the time of exposure to As (**Table 3.1**), with *BORON* transcripts 5-fold down-regulated at 1 d (**Fig. 3.3**), when As III concentration in the medium was the highest (**Fig. 3.2B**). In leaves, *BORON* transcripts are induced at 1 and 3 d in response to As (**Fig. 5.5B**), coinciding at 3 d with the highest B concentration (**Table 3.1**). Whereas Zn is described as an indispensible micronutrient, which mitigates As toxicity by modulating ROS and the antioxidant function in plants (Das et al. 2016) or by improving the thiol metabolism (Srivastava and Srivastava, 2017), no major changes were detected in Zn concentration apart from the increase at 1 d in As-exposed plants. With regard to Fe, our data showed that Fe translocation to leaves was more affected by As than any of the other elements, with an increased Fe concentration in roots exposed to As, whereas in leaves it decreased (**Table 3.1**). Shaibur et al. (2008) described that one of the symptoms of As toxicity is the formation of Fe plaques in roots and, as also seen in our case, Fe:P ratios in the roots of the As-exposed plants were higher than those observed in the control roots. This suggests that, in the liquid culture medium, As may have been adsorbed with Fe on the surface of the roots, forming Fe-As plaques. Thus, the iron plaque formed on the root surface will act as a natural As barrier and reduce As uptake by the plant and its translocation to shoots.

Besides the impact of other elements in the medium on As uptake, the speciation of As also plays an important role in the accumulation of As and tolerance by the plant (Moreno-Jimenez et al., 2010). The As was added to the culture medium as As V and after 24 h a 4% reduction to As III was observed (**Fig. 3.2B**). This chemical reduction can be attributed to metabolic activities of *S. atrocinerea* since no speciation was detected when the plant cuttings were not present in the culture medium. For this observation, two possible explanations can be given. On one side, plants might induce changes in the pH and in the redox potential of the culture medium as it was observed in this study (**Fig. 3.2A**), and those changes might affect the speciation of As. For an example, it has been proposed that protons released from organic acids (R-COOH) and excreted by plant roots may contribute to the reduction of As V to As III, while increasing the pH as the process consumes H^+ (Park et al., 2016). Interestingly, only As V was detected after 30 d and it matched with the highest pH value in the medium; whereas the highest As III concentration was detected

at 1 d, when the pH was the lowest (**Fig. 3.2A**). Therefore, another possible explanation for the presence of As III in the medium is a direct efflux of As III from the plant to the medium that can be linked to the proton gradient across the plasma membranes or dependent of the plant metabolism (e.g. direct As III from plant cells to the medium) (Xu et al., 2007; Park et al., 2016). Taking transcriptional regulations into account, since willow plants were able to induce the occurrence of As III in the medium, differences in transcript levels of genes encoding for As III transport were expected in roots of As-exposed plants.

In our case, we observed a noticeable up-regulation of the transcripts encoding the aquaporin NIP1.1, reported for As III uptake into the roots (Ma et al., 2008), and in transcripts for SIP1 at 1 d of As exposure (**Fig. 3.3**). This up-regulation diminishes over time, probably as a consequence of a very active As V reduction to As III during the first days of exposure, with a lot of free As III initially in the cytoplasm. This transcript up-regulation for As III transporters in roots, suggests that As V reduction has occurred even before its entry into the roots, which is supported by the presence of 4% of As III in the medium at 1 d of exposure (**Fig. 3.2B**), and which coincides with the highest up-regulation of *NIP1* and *SIP1*. In leaves, where As was mainly present as As V, no changes in transcript levels for the aquaporin transcripts were observed (**Fig. 3.5B**).

Once inside the cell, since As V has no affinity for the "-SH" groups in the PCs, the first step in As detoxification is As reduction (Finnegan and Chen, 2012). The main mechanism for As V reduction is the presence of As V reductases where GSH acts as electron donor (Dhankher et al., 2002). Arsenate reductases are believed to have evolved from the CDC-25 (cell division cycle) dual-specificity tyrosine phosphatases (Duan et al., 2007). Based on homology with the yeast As V reductase, ACR2P, Bleeker et al. (2006) identified a CDC25-like plant candidate and showed that it had arsenate reductase activity like it was also observed in other assays (Dhankher et al., 2006). In our study, As V reduction to As III was observed in roots right after As uptake with an increasing As III concentration in root tissues from 9% to 70% by the end of the study (**Fig. 3.2C**). This coincides with the *CDC25-1* up-regulation in roots of plants exposed to As (**Fig. 3.3**), whereas no changes were observed for *CDC25-2* (**Fig. 3.5A**).

Another mechanism to reduce As V in the plant is through a non-enzymatic reduction, where GSH is implied, but this process is relatively slow, so according to the NPT data we can attribute the large up-regulation observed in *GS* transcripts along the As-exposure time to PC production as a detoxification mechanism. This was reflected by the increased NPT concentrations in *S. atrocinerea* roots after As uptake (**Table 3.3**). Our results showed that although there was a clear up-regulation of *GS* transcripts in roots (**Fig. 3.3**), GSH concentrations of As-exposed plants remained constant over time and lower than those in

the roots of control plants (**Table 3.3**). However, since PCs use GSH as a building substrate, the decreasing concentrations of GSH are consistent with its use in PCs or other NPTs. This fast increase in NPT concentrations and the As III presence in the roots, support our observations of As speciation in the medium; where it seems that As III efflux by roots occurs right after As V uptake and that this efflux diminishes once the As III is complexed with thiols and stored in the vacuoles. Therefore, an increase in NPTs under increased As III presence points towards an As-PC complex formation possibly leading to less As III efflux. In support to this explanation, Raab et al. (2005) found that in sunflower roots the amount of As not complexed by thiols fell from 90% of total As after 1 h exposure, to 43% after 4 d of exposure. In addition, although PCs were synthesized in *S. atrocinerea* in response to As exposure and their concentration increased over time in roots (**Table 3.3**), there was only a slight increase, in transcripts coding for PCS at 1 d, similar to the behavior observed in transcripts of *GR* (**Fig. 3.5A**). This suggests that the induction of *PCS* expression is unlikely to play a significant role in regulating PC biosynthesis (Cobbett 2000). This agrees with Rea el al. (2004), who reported that PCS enzymes are expressed constitutively at relatively high levels and are generally unaffected by exposure of cell cultures or plants to heavy metal(loid)s. As described in other plant species, PC-based sequestration is considered to be essential for As tolerance, where hypertolerant ecotypes present higher PC concentrations under As exposure compared to non-tolerant ecotypes (Meharg and Hartley-Whitaker, 2002; Schat et al., 2002; Fernández et al., 2013). The 7-fold increase in NPT concentration observed in our case in roots of willow after 1 d of As exposure, it is then related to a fast As V reduction to As III and to the need to synthetize longer-chain PCs to chelate the increasing concentrations of As III, and therefore maintaining cellular stability. As it has been reported by Sharma et al. (2016), longer chain PCs contribute to a more effective cellular detoxification due to a higher metal-binding capacity and formation of more stable As-complexes that will prevent the interaction with sulfhydryl groups of other proteins and hence affect the metabolism.

We also observed that in the roots, the organ where more As was accumulated, a greater PCs synthesis was present than in leaves where As accumulation is lower. Another interesting observation of our study is the presence of many unknown thiol products. This is in accordance with the results of Li et al. (2004) in *Arabidopsis*, where As exposure resulted in the expression of many unknown thiol products, whereas cadmium induced higher increases in traditional PCs (PC$_2$, PC$_3$, PC$_4$).

Most of the As speciation experiments described in literature propose As III as being the predominant As form in leaves (Kertulis et al., 2005; Zhang et al., 2009; Yan et al., 2012; Park et al., 2016). However, in our case As V was the main As species observed in leaves

throughout the experiment (**Fig. 3.2C**). Despite As exposure caused *de novo* synthesis of desGly-PC$_2$ and the increase of desGly-PC$_4$ in leaves of As-exposed plants, a possible explanation for the lack of As III observed in leaves, with the exception at 3 d, could be attributed to the relatively low concentration of As in leaves as compared to roots (**Table 3.2)** , which might require a less effective NPT response. Another explanation could be related to the stability of the As-thiol complexes present in leaves, where As III could be mainly bound to GSH which was present at higher concentrations than in roots, and represent the main NPT in leaves (**Table 3.3**). Since As III – GSH complexes are less stable that As III – PCs, a dissociation of these complexes could take place with the consequent re-oxidation of As III to As V (Bluemlein et al., 2009; Zhao et al., 2009). In relation to this, the As V presence in leaves of *S. atrocinerea* might explain the need for the up-regulation of the CDC25-like tyrosine phosphatases pathway observed at 10 and 30 d, when As increased in shoots (**Fig. 3.5B**), and exceeded plant toxic limits (White and Brown, 2010). In contrast to PCs that rely on enzymatic synthesis, MTs, which are also important metal chelators in plant cells, are direct products of mRNA translation (Anjum et al., 2015). Examples of MTs induction under metal exposure in *Salix* have been described by Konlechner et al. (2013), and it is known that metals like Zn or Fe bind to MTs with the highest affinity (Blindauer et al. 2010). Therefore, in this study according to the differential transcription pattern of *MT1A* between roots and leaves, due to the Fe accumulation in roots and its reduction in leaves, it could be that *MT1A* induction in leaves (**Fig. 3.5B**) corresponds to the need of supplying enough Fe and that this up-regulation is not involved in direct As chelation. However, its induced expression in leaves forms part of the response to the As-induced stress.

Once As V is reduced to As III and complexed to NPTs to limit its toxicity, these complexes are taken up by ABC transporters and stored in the vacuole. ABC transporters constitute one of the largest protein families, present in organisms ranging from bacteria to humans, and have been identified as transporters involved in detoxification processes by transporting metal(loid)-PC complexes (Kang et al., 2011). It is known that As III-PCs complexes have a low stability and their storage into the acidic environment of vacuoles can limit its dissociation and As release back into the cytosol (Schmöger et al., 2000). Song et al. (2010) already emphasized that engineering of vacuolar PC transporters in plants may be of potential use in phytoremediation. According to this, in our study, the up-regulation observed of *WBABCT* transcripts (**Fig. 3.3**) highlights its role in metal(loid)-PC complexes transportation and constitutes an interesting target gene to increase accumulation for phytoremediation purposes. Interestingly, in leaves, since only As V was present and at low concentration compared to roots, no differential up-regulation of *WBACT* transcripts was observed as compared to control (**Fig. 3.5B**).

Taking into account that speciation and distribution of As in the plant can provide important information and help to understand the mechanisms for As accumulation, translocation, and transformation as noted by Zhang et al. (2002), our results suggest that the As tolerance mechanism of *S. atrocinerea* relies on As V reduction in roots but not in leaves. Therefore, limited As V translocation by an effective As V reduction to As III and its complexation to NPT compounds and further sequestration into the root vacuoles, as supported by the gene expression, seems to be the reason for the tolerance of *S. atrocinerea* to As.

Under As accumulation, stress is induced in plant cells. Although in leaves As detoxification processes are not really activated as seen in roots, *S. atrocinerea* plants respond to As in both roots and leaves by altering gene expression related to general stress. This response includes alterations at the transcript level of genes related to (1) cell wall synthesis, as a down-regulation of the cellulose synthase like A (*CSA*) (**Fig. 3.5A,B**) is observed; (2) ethylene biosynthesis, with *ACCS* notably up-regulated in roots and to a lesser extent in leaves, probably explained a low As translocation (**Fig. 3.4**); and (3) transcripts related to the synthesis of heat shock proteins, like *AIP-1*, a highly conserved gene selectively activated by As III in many cell types (Sok et al., 2001). In our experiment the highest up-regulation was observed in roots at 1 d after exposure and it decreased over time (**Fig. 3.4**). Since this protein is As III-induced, and As III concentrations increase over time in roots, this suggests an effective complexation of As III from 1 d on with NPTs to prevent its toxicity. Another As-related response of willow is the biosynthesis of phenylpropanoids that may culminate with the increased production of tannins (Yanitch et al., 2017) and might be an important stress defense mechanism in leaves. In our study, we observed an early up-regulation of selected genes (*CHS1, CHS2, 2HFLR* and *F3H*) in the flavonoid pathway at 1 d after As exposure (**Fig. 3.6 B** and **Fig. 3.5B**), whereas no major changes in the expression pattern or a down-regulation were noticed later on as compared to control conditions. Therefore, by the information provided by the transcript levels, we suggest that *S. atrocinerea* relies on the phenylpropanoid pathway to cope with As toxicity during the early times of exposure, but further investigation at metabolic level is essential.

3.5 CONCLUSIONS

The selected *S. atrocinerea* clone naturally growing in an As-contaminated brownfield showed great tolerance when grown in the presence of a high concentration of As and accumulated more than 2,400 mg As kg^{-1} dry weight in its roots without showing phytotoxicity symptoms. Our findings reveal that under the presence of As V in hydroponic

conditions, willow plants show a transcriptional regulation of genes involved in nutrient transporters, As V reduction, glutathione synthesis and sequestration of As into the vacuoles, together with genes involved in stress responses, which coincides with a rapid As III presence and accumulation in root tissues, altered nutrient profile and *de novo* synthesis and increase of NPT compounds, all of which contribute to the tolerance to the metalloid by *S. atrocinerea*.

The high As accumulation together with a high biomass yield makes this willow species a potential tool for its use in As phytoremediation. Overall, a better understanding of the physiological mechanisms of tolerance to arsenic toxicity in *S. atrocinerea* was achieved through this study by experimental verification of the significance of particular transcripts complemented by an integrative analysis of nutrient profile, As accumulation and speciation, as well as NPT compounds synthesis. However, according to our observations, further research should also focus on what happens in real polluted soils where, apart from As, there are usually other metal(loid)s at high concentrations that can affect the plant detoxification responses.

Chapter 4

Bacterial-induced Arsenic Speciation Affects As Accumulation and Tolerance in *Salix atrocinerea*

4 Bacterial-induced Arsenic Speciation Affects As Accumulation and Tolerance in *Salix atrocinerea*

4.1 INTRODUCTION

Soils polluted with metal(loid)s due to industrial activities are not only a major environmental but also an economical problem (Tchounwou et al., 2012), as they are not only unsuitable for food crop production but also unusable for industrial activity and, therefore are abandoned indefinitely (Tóth et al., 2016b). Weathering processes allow dispersion of toxic metal(loid)s, like arsenic (As), which could affect drinking water supply and surrounding land areas and as such cause an undesirable impact on the food chain and incur health risks as well as economic and social costs (Kaur et al., 2011; Gallego et al., 2016). Arsenic is a trace element found ubiquitously in the Earth's crust at an average concentration of approx. 6 mg Kg^{-1} (Kabata-Pendias, 2010). Although its abundance on the Earth's crust as compared to other elements places As on the 54th position, As can become concentrated in some parts of the world because of natural mineralization or industrial activities (Gallego et al., 2016). In soils, under aerobic conditions, the predominant form of As is arsenate (As V), whereas arsenite (As III) which is more toxic and mobile is more prevalent in anaerobic environments. Many reports have shown that in rice paddy fields, As III-oxidizing bacteria are able to detoxify As III by oxidizing it to As V, which is strongly retained into oxides/hydroxides of aluminum (Al), manganese (Mn) or iron (Fe) and aluminosilicates, making it less toxic and less soluble (Das et al., 2016). For phytoremediation of brownfields, however, the interest focuses on removing as much as possible of the metalloid from the soil, and therefore As V should be reduced to As III and thereby become more available for plant uptake.

To eliminate, reduce or make these toxic metal(loid)s less available, some techniques have been developed based on biological processes (Vangronsveld et al., 2009). One of these techniques is phytoremediation, defined as the use of plants to eliminate contaminants from the soil or to reduce their harmful effect (Salt et al., 1998). Phytoextraction is based on the use of plants and their associated microorganisms to absorb metal(loid)s from soil and translocate them to aboveground tissues, where they accumulate (Lombi et al., 2008). This technology is less expensive and harmful for the environment than the more conventional civil engineering methods, like soil excavation (Kidd et al., 2015). Afterwards, the plant material rich in metal(loid)s is picked up for its posterior treatment. In phytostabilization, plants accumulate metal(loid)s mainly in their roots and make them less bioavailable (Vangronsveld et al., 2009). Some plants produce root exudates that improve metal

immobilization and diminish the risk of toxic elements entrance into the food chain (Kumar et al., 2017).

Effectiveness of a phytoextraction process is strongly constrained by plant characteristics such as its capacity to accumulate and translocate metal(loid)s to their aboveground tissues, biomass, growth and an extension of the radical system (Maestri et al., 2010, Janssen et al., 2015). Most studies related to plant responses to metal(loid)s have been performed in model species like *Noccaea* or *Arabidopsis*. Nonetheless, these plants have low possibilities to be used in soil decontamination due their low biomass and lack of economic profit. Therefore, woody plants, such as willow, show various advantages in phytoremediation (Janssen et al., 2015) including a greater radical system that can reach more soil volume and therefore, limit notably pollutant entry into the environment (Mench et al. 2009). Although woody species show a lower phytoextraction rate than hyperaccumulating herbaceous species, the fact that they produce a greater biomass results finally in a higher total metal(loid)s extraction (Greger and Landberg, 1999). Furthermore, woody plants can afterwards be processed as biofuel or in lumber industry with the subsequent economic profit (Rockwood et al., 2004; Thewys et al., 2010; Witters et al., 2012b).

Arsenate acts as a phosphate analogue and is taken by the plant through the same transporters as phosphate, which impairs phosphate metabolism (Shin et al., 2004). Arsenite enters through aquaglyceroporins (Ma et al., 2008) and binds to sulfhydryl groups, "-SH", of amino acids (Ehlrich, 1990) such as cysteine in proteins, inactivating in this way a wide range of metabolic enzymes (Ahsan et al., 2008). Although As III is more toxic than As V, As V or As III provoke similar toxic effects, because once inside the plant cells, As V can be reduced to As III by arsenate reductases (Oden et al., 1994). Arsenic accumulation in plant tissues is a stress factor for the plant because it alters the cellular homeostasis and increases the amount of reactive oxygen species (ROS) that must be kept in balance by the antioxidant defense systems (Birben et al., 2012). Non-enzymatic antioxidant defense systems to neutralize ROS include the compounds proline, glutathione (GSH), carotenoids and phenolic compounds (Abbas et al., 2018). Besides this, As can also be bound to chelating compounds (Hartley-Whitaker et al., 2001b; Batista et al., 2014) and stored into the vacuoles (Song et al., 2010). The heavy metal-binding ligands in plants are phytochelatins (PCs) and metallothioneins (MTs), which are different classes of cysteine-rich protein molecules (Cobbett and Goldsbrough, 2002). PCs are a family of non-protein thiols that act as chelators and are made of increasing repetitions of the dipeptide ɣ-glutamyl-cysteine with glycine as terminal amino acid. In the (ɣ-Glu-Cys)$_n$-Gly polymer, where n = 2-11, As binds to the thiol group from cysteine (Hirata et al., 2005). PCs have been identified in a

wide range of plants, fungi and bacteria and they are synthesized from GSH by the enzyme phytochelatin synthase, a constitutive mechanism in higher plants, which requires activation by metal(loid)s, being Cd the most effective inducer (Clemens, 2006). Interestingly, PC molecules not only have high metal-binding capacity but also strong scavenging activity against ROS (Tsuji et al., 2002). MTs are also important metal chelators in plant cells, but in contrast to PCs, are encoded by genes and thus are products of mRNA translation (Anjum et al., 2014).

Endophytic bacteria are bacteria isolated from inner plant tissues after sterilization of their surface and that do not damage the host plant (Sturz et al., 2000). These bacteria can also be present in the rhizosphere and in the other way around (Reinhold-Hurek and Hurek, 1998; Berg et al., 2005). Plant-associated bacteria can generate drastic physiological changes in nutrient availability that affect plant growth and development (Conrath et al., 2006; Hardoim et al., 2008). Endophytic as well as rhizosphere bacteria can synthesize plant hormones such as the indole-3-acetic acid (IAA), gibberellins (GAs) and cytokinins (CKs) that can affect plant growth and development (Glick, 2012). Bacteria could also block efficiently the stress caused by ethylene since it has been reported that some plant-associated bacteria contain an enzyme, 1-aminocyclopropane-1-carboxylate deaminase, that catalyses the cleavage of 1-aminocyclopropane-1-carboxylate, the immediate precursor of ethylene in plants (Glick et al., 1998).

Microbial processes or metabolites produced by soil or rhizosphere bacteria affect the phytoremediation of metal(loid)s by changing their redox state and, thus, their solubility and availability for plant uptake (Kuffner et al., 2010). Bacterial metabolites such as siderophores, auxins, ethylene blockers or phosphate solubilizers can enhance plant growth and biomass in polluted areas and therefore, can favor contaminant removal by the plant and accelerate the phytoremediation process (Luo et al., 2011). Both As V and, especially, As III are toxic to bacteria but some species have developed ways to survive in As-rich environments (Hoeft et al. 2010). It is known that most bacteria reduce As V to As III inside the cells through the action of the *ars* operon and exclude As III via an As III efflux pump (Zhao et al., 2015; Yang et al., 2012). Arsenic reduction minimizes As V competition with P uptake so the cells can maintain normal growth and metabolism (Ghosh et al. 2015). As III can also enter the cell through aquaporins and be methylated and immobilized in the bacterial biomass (Oremland and Stolz, 2003).

Up to date, most of the published research is focused on studying the separated, independent effect that microbial communities and plants have on contaminant uptake, degradation and accumulation. Nonetheless, plant-bacteria interactions play a crucial role

in plant development and metal(loid)s availability and toxicity. This information is even scarcer for arsenic, including the potential As bacterial transformations in speciation. It has been recently observed that the inoculation of plants with arsenic-resistant, plant-growth-promoting bacteria that can reduce As V to As III, particularly bacteria that are indigenous to polluted sites, can improve the efficiency of As phytoextraction (Lampis et al., 2015; Mesa et al., 2017). With the aim of deepening into the scientific basis of the processes that accompany the development of the microbiome of woody plants and their mutual influence, we designed *in vitro* bioaugmentation experiments with a *Salix atrocinerea* clone isolated from an As-field, inoculated with the endophytic bacterium *Pantoea* sp. or the rhizosphere bacterium *Rhodococcus erythropolis*, both selected by their plant growth-promoting traits and high As-reducing capacity. In order to uncover the role that *S. atrocinerea*-associated bacteria play in As accumulation and tolerance in the plant, we analyzed changes in the accumulation of As, its speciation, the production of PCs, the immobilization of As and the expression of several genes in an *in vitro* system. The data here obtained will contribute to optimize the phytoremediation practices with a more rational basis.

4.2   MATERIALS AND METHODS

4.2.1   *In vitro culture and bacteria treatments*

Non-lignified and aseptic juvenile *S. atrocinerea* plants of 12-15 cm length, previously propagated by *in vitro* techniques from a willow tree growing in the multi-metal-polluted Nitrastur brownfield (Asturias, Spain) were placed inside sterilized polycarbonate magenta vessels of 600 mL capacity (Magenta Corp. USA). Vessels contained 10 cellulose plugs disposed on the base to hold the plant and 80 mL of Woody Plant Medium (WPM) (Lloyd 1981), pH 5.7, with 0.2 g L^{-1} of sequestrene 138-Fe (Ciba-Geigy AG), 30 g L^{-1} of sucrose, and supplemented with 0 and 18 mg L^{-1} As. The As was added as sodium heptahydrate arsenate ($Na_2HAsO_4.7H_2O$). This As concentration was similar to that found at the exchangeable fraction of the Nitrastur brownfield soil. Since a possible conversion of As V to As III in bacterial inoculated treatments could occur, an extra arsenite treatment (added as 5 mg L^{-1} $NaAsO_2$) was performed to establish a reference for *S. atrocinerea* response to this As species, where As III was added in equivalent toxic concentrations (5 mg L^{-1}) to As V according to previous lab results (data not shown).

The bacteria strains *Pantoea sp.* (AV62) and *Rhodococcus erythropolis* (AV96), isolated respectively from endophytic and the rhizosphere environment of *S. atrocinerea*

plants growing on the As-polluted brownfield with plant-growth-promoting characteristics and high As-resistance [*Pantoea* sp. up to 50 mM As V and 20 mM As III, and *R. erythropolis* up to 100 mM As V and 20 mM As III, both in 284 liquid medium (Schlegel et al.,1961)], were grown separately in 500 mL Erlenmeyer flasks containing 100 mL of 869 medium (Mergeay et al., 1985) under continuous shaking at 30 °C to reach 10^9 colony-forming unit (cfu) mL^{-1} (24 - 48 h). Bacterial cells were recovered by centrifugation at 8,000 rpm for 10 min at 4 °C and the bacterial pellets were re-suspended in WPM liquid medium to minimize changes in nutrient media solution after inoculation. Then, the bacterial suspension was pipetted to the medium of each plant until a concentration of 10^4 cfu mL^{-1} was obtained. An excess of liquid volume was corrected in the non-inoculated treatments. Plants were randomly placed in a culture chamber with controlled environment at 25 °C, 150 µmol m^{-2} s^{-1} and 16-h photoperiod.

A total of 5 treatments were generated:

−*S. atrocinerea* plants not exposed to arsenic, control.

−*S. atrocinerea* As-exposed plants to 18 mg L^{-1} As V, As V.

−*S. atrocinerea* As-exposed plants to 5 mg L^{-1} As III, As III.

−*S. atrocinerea* As-exposed plants to 18 mg L^{-1} As V and inoculated with 10^4 cfu mL^{-1} *Pantoea sp.*, As V + P.

−*S. atrocinerea* As-exposed plants to 18 mg L^{-1} As V and inoculated with 10^4 cfu mL^{-1} *R. erythropolis*, As V + R.

After 30 days, plants were carefully removed from the magenta vessels and the roots were thoroughly rinsed with tap water and three times with double de-ionized water (Milli-Q 185 Plus System) at 4 °C. Shoots were rinsed only once in distilled water. To determine the influence of the treatments on plant growth, fresh and dry weights as well as lengths of shoots and roots were measured. Afterwards, plants were separated into shoots and roots and samples of at least 4 different plants were analyzed individually for each treatment. Plant material was homogenized with liquid nitrogen and stored at −80 °C until use.

4.2.2   *Analysis of elemental nutrients, arsenic and arsenic speciation in plant*

The nutrients boron (B), copper (Cu), Fe, P, and zinc (Zn) together with As concentrations were determined in shoots and roots of *S. atrocinerea*. For this, 100 mg of dry powdered and processed as described in ***section 2.3.1.***

To determine the As speciation in shoots and roots, 100 mg of finely-ground sample in 2.5 mL of 0.3 M nitric acid solution and analyzed as described in ***section 2.3.1.***

4.2.3   *Analysis of photosynthetic pigments, hydrogen peroxide, lipid peroxidation and free proline*

Chlorophylls and carotenoids were extracted from 100 mg of frozen shoots using 10 mL of 80% acetone. In addition, 100 mg of frozen shoots were extracted in 1% (v/v) hydrochloric acid dissolved in ethanol for anthocyanins determination and after centrifugation at 16,000 g for 5 min at 4 °C, 1.2 mL chloroform was added to 600 μL of supernatant. Homogenates were centrifuged at 3,000 g for 10 min before measuring the absorbance. The pigment content was calculated according to the equations of Porra (Porra 2002), Lichtenthaler and Wellburn (1983) and Lambert- Beer law for anthocyanins (Vanderauwera et al., 2005; Porter et al., 2009).

For the hydrogen peroxide (H_2O_2) content determination, 100 mg of frozen shoots were mixed with 2.5 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 10,000 g for 15 min, following the method previously described (Fernandez et al., 2013). To a 0.5 mL of the supernatant, 1 mL of 1 M KI and 0.5 mL of 100 mM phosphate buffer (pH 7.6) were added. The absorbance of samples was measured at 390 nm and the H_2O_2 content was determined using a calibration curve and expressed as μmol H_2O_2 g^{-1} fresh weight (FW). Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) (Demiral and Türkan, 2005; Carrasco-Gil et al. 2012). MDA was extracted from 100 mg of frozen shoots with 2.5 mL of 0.1% TCA and the homogenates centrifuged at 10,000 g for 15 min. To a 0.5 mL of the supernatant, 2 mL of 20% TCA with 0.5% TBA was added and the mixture heated at 95°C for 30 min. Then, samples were quickly cooled in ice and centrifuged at 10,000 g for 15 min. The absorbance of the supernatant was measured at 535 nm (ε = 155 $mM^{-1}cm^{-1}$). The absorbance at 600 nm was used to correct the non-specific turbidity. MDA content was expressed as nmol g^{-1} FW. Free proline content was determined according to the methods described in (Bates, Waldren and Teare 1973; Demiral and Türkan, 2005). A 100 mg aliquot of fresh leaf material was homogenized in 3% (w/v) sulfosalicylic acid and centrifuged at 10,000 g for 15 min. A 1 mL aliquot of supernatant was mixed with 1 mL of glacial acetic acid and acid ninhydrin (1:1, v/v). The mixture was heated for 1 h at 100 °C in a water bath and cooled quickly on ice to stop the reaction. Then proline was extracted in 5 mL toluene and the absorbance read at 520 nm. Free proline concentration was determined using a calibration curve and expressed as μmol g^{-1} FW.

4.2.4 Analysis of non-protein thiols

The extraction and analysis of non-protein thiols (NPTs) were carried out in shoots and roots of *S. atrocinerea* from 150 mg fresh samples and following the protocol described in **section 2.3.3.**

4.2.5 Analysis of total flavonoids, condensed tannins and total phenols

The samples were processed by the method described in Nair et al. (2012). One g FW was extracted with 15 mL of methanol and the residue extracted with another 15 mL methanol. The combined methanolic extracts were filtered and evaporated at 35 °C under reduced pressure, re-dissolved in methanol at a concentration of 10 g L^{-1}.

The total flavonoid content was determined using the method of Meda et al. (2005). The reaction and absorbance measure were carried out in 96-well microplates. In brief, 150 µL of 2% AlCl$_3$ was mixed with the same volume of plant extract. Absorbance reading at 415 nm was taken after 10 min against a blank sample consisting of 150 µL of sample solution and 150 µL of methanolic extract without AlCl$_3$. The total flavonoid content was determined using a standard curve of quercetin at 5–55 µg mL^{-1}. The average of three readings was used and then expressed as quercetin equivalents (QE) on a FW basis.

Condensed tannins were estimated by the vanillin method (Sun et al., 1998). The reaction in 96-well microplate consisted of 60 µL of sample or standard, 120 µL of 1 % (m/v) vanillin solution in methanol and 120 µL of 10 % (v/v) H$_2$SO$_4$ solution in methanol. The reaction temperature was 30 °C, the reaction time 15 min, and the absorbance was measured at 500 nm. (+)-Catechin was used for standard curve at 0.015–0.5 g L^{-1}, and the results were expressed as mg of (+)-catechin equivalents (CE) g^{-1} FW.

For total phenolic content quantification, accomplished in 96-well microplate, a 10 µL aliquot of the methanolic extract solution was diluted with 215 µL of H$_2$O and 15 µL Folin–Ciocalteu reagent and 60 µL of 20 % (m/v) Na$_2$CO$_3$ solution for 30 min at 25 °C. Absorbance was measured at 750 nm. Gallic acid was used to calculate the standard curve at 0.025–0.6 g L^{-1}, and the results were expressed as mg of gallic acid equivalents (GAE) g^{-1} FW.

4.2.6 Gene expression analysis

Isolation of RNA was carried out using the protocol described by Chang et al. (1993) with slight modifications. Frozen shoots or roots samples of 100 mg were homogenized and processed as described in **section 3.2.4**

Reverse Transcription quantitative PCR (RT-qPCR) was performed with an ABI Prism 7900HT Fast Real Time PCR system (Applied Biosystems), using Fast SYBR Green chemistry. Gene primers (**Supplementary Table 1**) were designed using Primer 3 (Untergasser et al., 2012), according to sequences of genes obtained in the Phytozome nucleotide database and described in **section 3.2.4.**

In roots *ARF2*, *α-TUB2* and *DNAJ* were the three most stable reference genes in all the sample sets according to GrayNorm algorithm and the combination of the three was used for normalization. In shoots a combination of *ACT7*, *α-TUB2* and *DNAJ* was used for normalization.

Bacteria presence in plant tissues was determined by relative expression of 16S rRNA's bacterial transcripts, for which primers were designed to avoid unspecific amplification of plant DNA, as well as to generate shorter PCR products appropriate for qPCR as described above. The qPCR of 16S rRNA gene was also performed in the same way as described above. The primer sets for *Pantoea* sp. (F: tgcatttgaaactggcaggc, R: agcgtcagtctttgtccagg) and for *R. erythropolis* (F: cgtgtctcagtcccagtgtg R: cgtcgtttgtgaaaaccagca) and had a 101% efficiency ($R^2 = 0.999$) and 94% efficiency ($R^2 = 0.998$), respectively.

4.2.7   Analysis of plant growth regulators (PGRs)

For the extraction and purification procedures for PGRs used a modification of Pan et al. (2008, 2010) as described in Delatorre et al., (2017). One mL of the extraction solution consisting of 2-propanol:H_2O:HCl (2:1:0,002 v/v/v) including IDS (10 ng of BA-d_7, 20 ng of ABA-d_6, DHZ-d_3, IAA-d_5 and SA-d_6; 40 ng of Bk-d_5 and GA$_7$-d_2) was added to the samples in PTFE tubes. The mixture was agitated for 30 min at 4 °C after which 1.8 mL dichloromethane was added and the mixture was agitated again for another 30 min at 4 °C. After the agitation step, the solvent formed two layers separated by plant debris. The lower layer, which contained all the compounds of interest, was collected. The upper layer was discarded and the resultant pellet was re-extracted again following the same procedure. The lower layer was again collected and combined with the previous one. After that, the combined lower layers were concentrated to dryness in 2 mL glass vials under nitrogen stream and stored until analysis at −20 °C.

Dried samples were re-suspended in a final volume of 200 μL of 100% methanol and filtered through a 0.2 μm regenerated cellulose filter (Captiva Premium Syringe Filter Regenerated Cellulose, Agilent Technologies, Madrid, Spain) loaded with 15 mg of silica (SiO_2) (Bondesil C18 bulk sorbent, 40 μm, 100 g, Agilent Technologies, Madrid, Spain).

Chromatographic separation was carried out in a reverse phase column (Zorbax SB-C18 2.1 × 50 mm) kept at 40 °C and coupled to a Zorbax Eclipse Plus C18 2.1 × 5 mm precolumn (Agilent Technologies, Madrid, Spain). Two solvents were used as mobile phases at a flow rate of 0.45 mL/min: acidified MeOH with 0.1% formic acid (solvent A) and ultra-purified water adjusted to pH 4 with formic acid (solvent B), both of them buffered with ammonium formate (10 mM). A linear gradient of MeOH was used for analyte elution: 10–50% in 7 min, then reaching 100% in 2 min which was maintained for 2 more minutes. After this, initial conditions were restored over a period of 1 min and held for a further 6 min, allowing the column to equilibrate, resulting in a total time of 18 min per sample. The injection volume was 5 μL. Analysis of the compounds was based on appropriate precursor-to-production transitions. Diagnostic of precursor-to-product ion transitions, optimized conditions and quantification of PGRs by means of calibration curves is described in Delatorre et al. (2017).

4.2.8 Statistical analysis

To evaluate the effects of the different treatments on *S. atrocinerea* for the measured variables, a one-way or a two-way Analysis of variance (ANOVA) was performed. Data were treated and transformed when necessary as described in **section 3.2.5**. In the gene analysis the previous was performed on both the normalized and the non-normalized data, although only the first are presented both were taking into account to establish the significance of the results. Results are expressed as the mean ± standard deviation of at least three independent replicates. All data were analyzed using R (version 3.3.1, http://www.r-project.org/) with the packages mixOmics (for PCA, version 6.0.1, http://www.mixOmics.org) and agricolae (version 1.2e4, http://tarwi.lamolina.edu.pe/~fmendiburu). Outliers were determined using the Extreme Studentized Deviate method (GraphPad Software, La Jolla, CA, USA) at significance level p ≤ 0.05.

4.3 RESULTS

4.3.1 *Bacterial and plant growth and nutrient analysis*
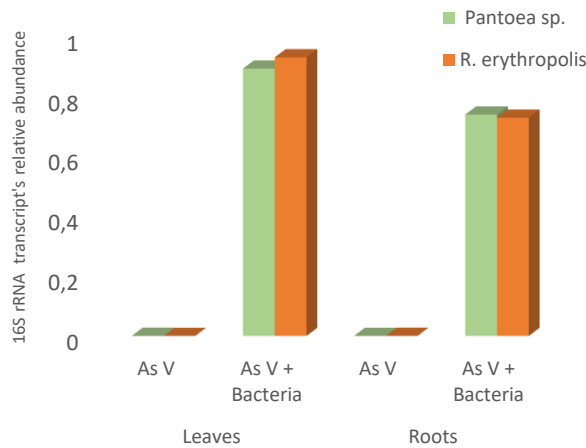
Fig. 4.1. Relative abundance of 16S rRNA transcripts after primer adding and qPCR amplification of As V and As V + P/ As V + R plant samples of at least four biological replicates, each containing at least one individual plant.

Regarding the bacterial growth, the starting bacterial cell concentration of inoculated treatments (As V+ P and As V + R) was 10^4 cfu ml^{-1} and after 30 days it increased up to 10^8 cfu ml^{-1} for *Pantoea* sp. and 10^9 cfu ml^{-1} for *R. erythropolis*. Both bacteria were able to efficiently colonize roots and shoots, as proven by the presence of their 16S rRNA markers in the corresponding plant extracts (**Fig. 4.1**)

After 30 days of culture, no external symptoms of phytotoxicity, nor decrease in root or shoot length or biomass decrease measured as fresh and dry weight (DW) were observed in As-exposed plants except for those inoculated with *Rhodococcus erythropolis* (As V + R) (Fig 1A, 1B, 1C). In this
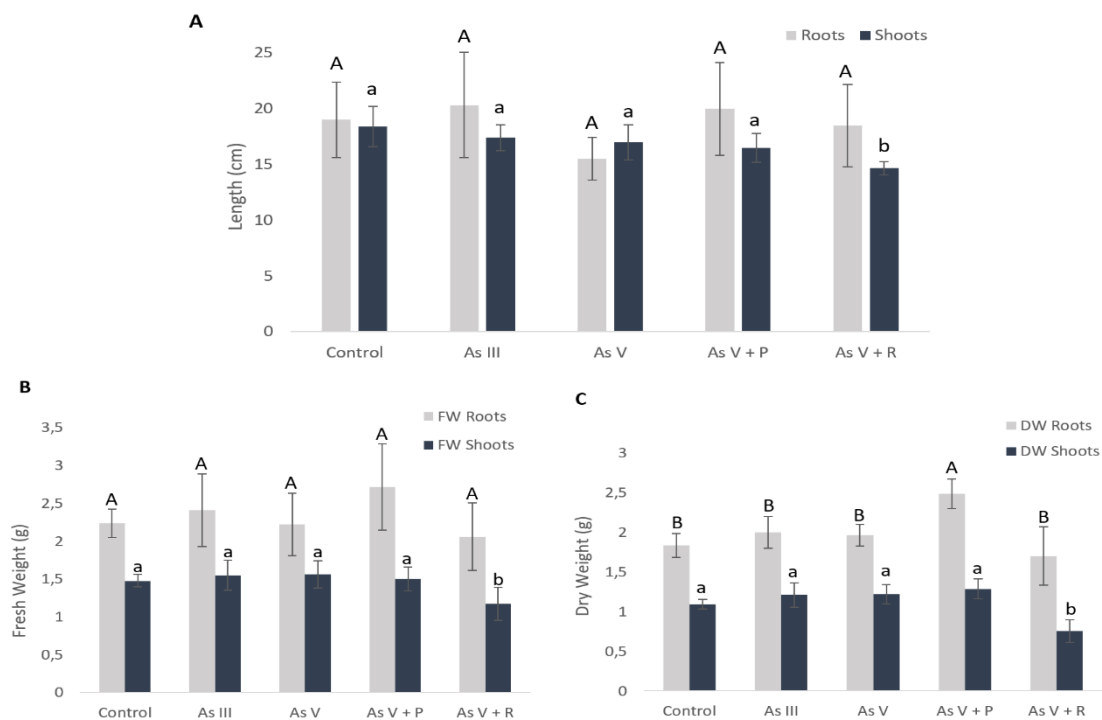
Fig. 4.2. Length (**A**), fresh weight (**B**) and dry weight (**C**) of roots and shoots of *S. atrocinerea* at 30 days of culture under different treatments. Different letters (upper case for comparison within roots and lower case for comparison within leaves) indicate significant differences among treatments on HSD test at p < 0.05.

condition, some roots had a necrotic appearance and shoots were a bit reddish and we observed a 30% decrease in shoots biomass (**Fig. 4.2C**). In plants inoculated with *Pantoea* sp. (As V + P) an increase in root biomass was observed (**Fig. 4.2C**).

In roots, regarding nutritional composition, all As treatments showed lower P concentrations than in the control plants (**Table 4.2**). Fe, Cu and Zn concentrations were always higher than in shoots and their concentration in plants of As V and As III treatments did not differ from control plants (**Table 4.2**). However, the lower Fe levels were measured in roots of As V + P plants, while the highest concentration was detected in As V + R plants and a similar pattern was observed for Cu and Zn in bacterial-inoculated plants (As V + P and As V + R). Besides, in As V + R plants the Zn concentration exceeded not only those of the rest of the treatments but also that in leaves of As V + R plants (**Table 4.2**).

In shoots, a slight decrease in P concentration was observed in plants As V treatment, whereas the bacterial treatments showed the lowest P concentrations as compared to control (**Table 4.2**). In all the As treatments, Fe concentrations, especially in As V + R plants, were lower than in control plants (**Table 4.2**). No differences were observed for Cu and Zn concentrations between non-inoculated roots of plants exposed to As and roots of control plants, whereas they were reduced in shoots in inoculated plants (**Table 4.2**).

4.3.2 *Accumulation and As speciation in the culture medium and plant tissues*

After 30 days of culture, As-exposed roots of inoculated plants with *Pantoea* showed the highest As accumulation, followed by As V treatment (**Table 4.3**). In shoots, the highest As accumulation was observed in non-inoculated As V-exposed plants (As V), followed by inoculated plants (As V + P and As V + R) and As III-exposed plants (As III) (**Table 4.3**).

With regard to As speciation we observed that in the medium, although only arsenate was applied, a 2%, 10% and 93% of arsenite was measured in As V, As V + P and As V + R treatments, respectively (**Fig. 4.3B**). However, no spontaneous arsenic speciation was detected in the medium without plants or bacteria growing inside (data not shown). In the As III treatment a 27% of arsenate was detected in the medium (**Fig. 4.3B**). In As III and As V + R treatments, the pH of the medium was above 4, whereas in the control, As V and As V + P treatments, the pH of the medium was below 4, with the lowest value registered in As V + P (**Fig. 4.3B**).

Table 4.2. Nutrient accumulation (mg kg^{-1} DW) in roots and shoots of *S. atrocinerea* at 30 days of culture under different treatments. Different letters within the same row indicate significant differences among treatments on HSD test at $p < 0.05$.

Organ		Treatment				
		Control	As III	As V	As V + P	As V + R
Roots	P	3339.05 ± 75.74 a	3072.14 ± 50.38 b	3054.71 ± 47.03 b	2531.82 ± 61.24 c	1827.92 ± 4.30 c
	Fe	1364.24 ± 118.67 b	1460.47 ± 96.54 b	1493.96 ± 147.96 b	1092.90 ± 89.83 c	2035.67 ± 174.44 a
	Cu	12.00 ± 0.17 b	11.83 ± 0.13 b	12.78 ± 0.15 b	9.93 ± 0.05 d	13.72 ± 0.10 a
	Zn	78.05 ± 10.53 b	89.07 ± 12.52 b	80.84 ± 10.38 b	60.74 ± 4.44 c	144.27 ± 23.02 a
Shoots	P	4448.30 ± 104.40 a	4473.24 ± 195.99 a	4173.30 ± 108.36 b	2935.16 ± 79.52 c	2421.45 ± 67.75 c
	Fe	140.72 ± 13.22 a	107.91 ± 4.98 bc	111.43 ± 5.24 b	100.26 ± 2.95 c	69.05 ± 0.86 c
	Cu	5.19 ± 0.25 a	4.79 ± 0.43 a	5.66 ± 0.42 a	3.44 ± 0.18 b	2.82 ± 0.09 b
	Zn	137.88 ± 14.61 b	156.35 ± 14.35 b	180.95 ± 17.57 a	121.19 ± 11.25 b	86.80 ± 10.83 c

Table 4.3. Arsenic accumulation (mg kg^{-1} DW) in roots and shoots of plants of *S. atrocinerea* at 30 days of culture under different treatments. Different letters within the same row indicate significant differences among treatments on HSD test at $p < 0.05$. nd: not detected

As	As III		As V		As V + P		As V + R	
	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots
As III	319.00 ± 2.34 c	nd	639.53 ± 6.71 b	2.43 ± 0.08 f	800.14 ± 15.12 a	0.29 ± 0.00 g	195.23 ± 5.34 d	4.75 ± 0.20 e
As V	423.95 ± 9.33 c	7.89 ± 0.21 h	581.01 ± 5.83 a	40.75 ± 1.05 e	502.97 ± 8.29 b	23.25 ± 1.14 f	338.55 ± 7.20 d	17.87 ± 0.97 g
Total As	742.95 ± 17.10 c	7.89 ± 0.21 g	1220.54 ± 30.58 b	43.13 ± 0.69 e	1342.11 ± 29.87 a	23.24 ± 2.04 f	533.78 ± 7.32 d	22.62 ± 0.99 f

In plant tissues the As was detected as As III or As V but no methylated As species were observed. In roots, arsenic was mainly present as As III in As V and As V + P plants (**Fig. 4.4.3C**), whereas in As III and As V + R plants, As was mainly as As V. In shoots, arsenic accumulated mainly in the form of As V in all assayed treatments (**Fig. 4.3C**). Interestingly, no As III was found in shoots of As III-treated plants and a 6%, 1% and 21% was found in shoots of As V, As V+ P and As V+ R plants, respectively (**Fig. 4.4.3C**).
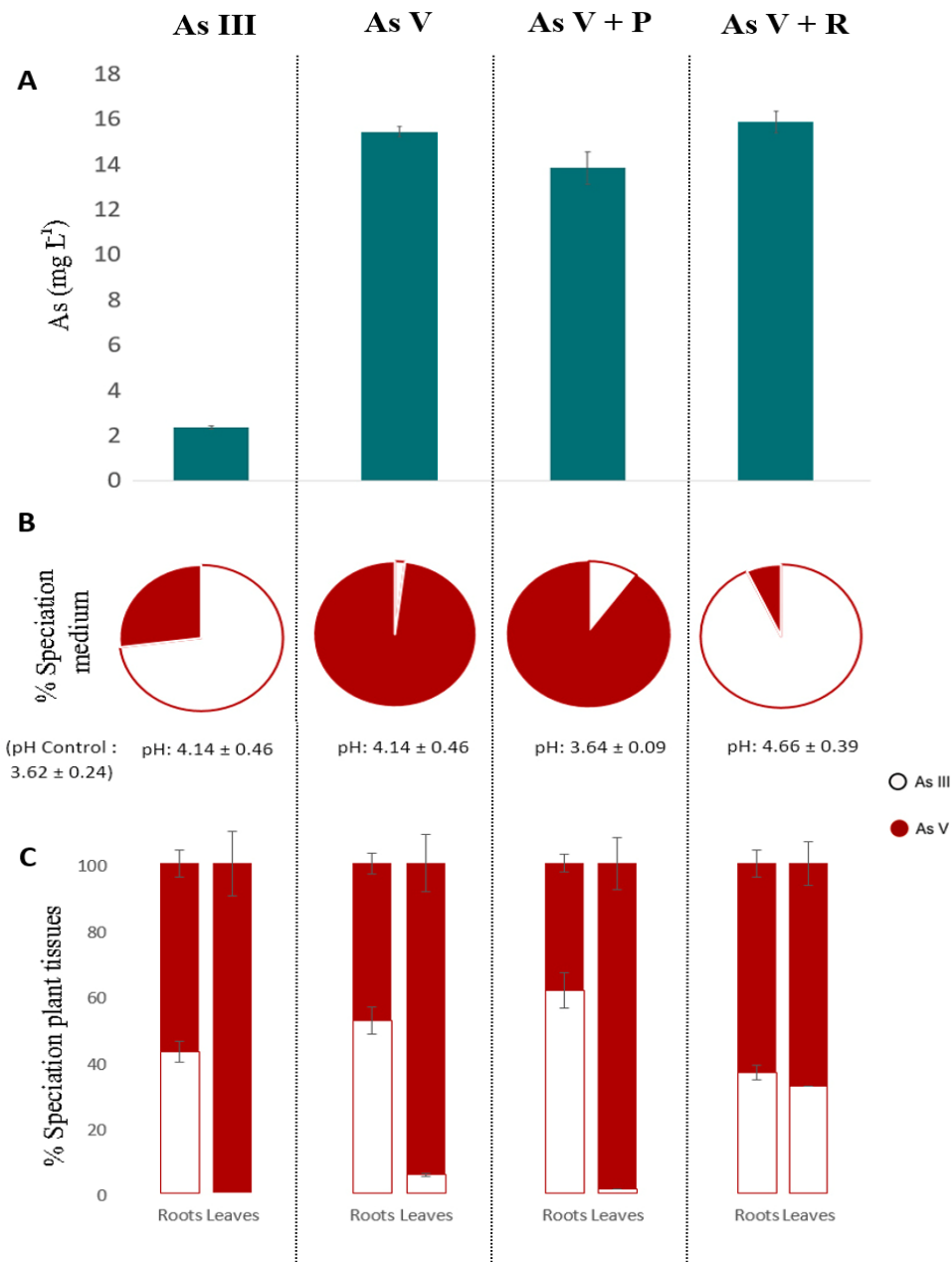

Fig. 4.3. As present in the medium (**A**) pH and percentage of As III and As V in the culture medium (**B**) and in roots and shoots (**C**) of *S. atrocinerea* at 30 days of culture under different treatments.

4.3.3   Analysis of photosynthetic pigments

In shoots of plants cultured in As III or inoculated (As V + P and As V + R), there was a decrease in chlorophyll *a* and *b* content (**Table 4.4**). No significant differences were observed between control plants and plants exposed to As V. Carotenoid concentration increased in all As treatments as compared to control plants. With regard to anthocyanins, increased concentrations in shoots of As V + P plants and especially in As V + R plants was observed when compared to control plants (**Table 4.4**).

Table 4.4. Photosynthetic pigments and anthocyanins concentration (mg g^{-1} FW) in leaves of *S. atrocinerea* at 30 days of culture under different treatments. Different letters within the same column indicate significant differences at p < 0.05.

Treatment	Chlorophyll *a*	Chlorophyll *b*	Carotenoids	Anthocyanins
Control	4.74 ± 0.27 a	2.60 ± 0.04 a	0.63 ± 0.00 c	0.32 ± 0.00 a
As III	3.61 ± 0.13 c	1.82 ± 0.10 b	0.69 ± 0.01 b	0.33 ± 0.01 a
As V	4.43 ± 0.16 a	2.47 ± 0.11 a	0.68 ± 0.00 b	0.32 ± 0.00 a
As V + P	3.94 ± 0.18 b	1.91 ± 0.01 b	0.67 ± 0.02 b	0.96 ± 0.02 b
As V + R	3.42 ± 0.17 c	1.36 ± 0.07 c	0.93 ± 0.00 a	1.76 ± 0.03 b

4.3.4   Parameters related to oxidative stress

Roots of As-exposed *S. atrocinerea* showed a decrease in the H_2O_2 concentration (**Table 4.5**), whereas in shoots, there was an increase in the H_2O_2 concentration in As III, As V + P and, especially, in As V + R plants (**Table 4.5**). The malondialdehyde (MDA) concentrations in roots and shoots were similar in all treatments assayed except for As V + R, where MDA reduced to half in roots, whereas in shoots it doubled (**Table 4.5**). With regard to proline concentration, it was under the detection limit in roots of every treatment, whereas in shoots it was doubled in As V + R plants (**Table 4.5**).

Table 4.5. Hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and free proline in leaves of *S. atrocinerea* at 30 days of culture under different treatments. Different letters within the same column indicate significant differences at $p < 0.05$. nd: not detected

Organ	Treatment	H_2O_2 ($\mu mol\ g^{-1}$ FW)	MDA ($nmol\ g^{-1}$ FW)	Free Proline ($\mu mol\ g^{-1}$ FW)
Roots	Control	3.16 ± 0.27 a	6.38 ± 0.36 a	nd
	As III	1.34 ± 0.19 c	7.59 ± 0.82 a	nd
	As V	1.21 ± 0.23 c	7.69 ± 0.78 a	nd
	As V + P	1.05 ± 0.23 d	7.68 ± 2.46 a	nd
	As V + R	1.70 ± 0.14 b	3.44 ± 2.10 b	nd
Shoots	Control	3.84 ± 0.25 d	14.37 ± 0.91 b	24.22 ± 8.47 b
	As III	4.76 ± 0.17 c	14.22 ± 0.59 b	24.36 ± 7.49 b
	As V	3.42 ± 0.15 d	13.70 ± 2.05 b	21.03 ± 8.27 b
	As V + P	6.88 ± 0.13 b	12.39 ± 1.14 b	16.58 ± 9.84 b
	As V + R	9.50 ± 0.23 a	27.66 ± 3.01 a	45.89 ± 9.25 a

4.3.5   *Analysis of non-protein thiols*

Changes in the concentration of non-protein thiols (NPTs) in roots and shoots of *S. atrocinerea* were observed among treatments (**Table 4.6**, **Table 4.7**). In the roots of As-treated plants the concentration of NPTs was higher than in control plants and this increase varied widely among treatments as compared to the controls (from 2 fold in As V + R, up to 13 fold in As + P) (**Table 4.6**). In roots of control plants, cysteine (Cys) and glutathione (GSH) were the only NPTs observed and both thiols were present at higher concentrations than those seen in any other of the As treatments except in As V + R plants, where Cys levels were the highest (**Table 4.6**). In plants exposed to As, the increase of NPTs in roots was due to *de novo* synthesis of PC_2, desGly-PC_2, Cys-PC_2, PC_3, desGly-PC_3 and unidentified thiol compounds, named as TC (TC_{1-5}), with PC_3 and desGly-PC_3 showing the highest concentrations (**Table 4.6**). Noticeably, TC_5 was only present in roots of arsenic-inoculated plants (As V + P and As V + R) and at high concentrations, especially in the roots of plants inoculated with *Pantoea* sp. (**Table 4.6**)*.*

Table 4.6. Non-protein thiolic peptides (nmol GSH g^{-1} FW) in roots of *S. atrocinerea* at 30 days of culture under different treatments. Different letters for each row indicate significant differences among treatments on HSD test at $p < 0.05$. nd: not detected.

Thiol	Treatment				
	Control	As III	As V	As V + P	As V + R
Cys	5.41 ± 0.16 b	4.62 ± 0.13 c	2.83 ± 0.08 d	1.93 ± 0.06 e	7.35 ± 0.21 a
GSH	15.22 ± 0.44 a	6.87 ± 0.20 c	4.66 ± 0.14 d	7.35 ± 0.21 b	0.84 ± 0.02 e
PC_2	nd	11.15 ± 0.32 c	14.39 ± 0.42 b	16.77 ± 0.49 a	0.78 ± 0.02 d
desGly-PC_2	nd	nd	1.30 ± 0.06 a	1.20 ± 0.04 a	nd
TC_1	nd	1.42 ± 0.04 b	2.67 ± 0.08 a	nd	nd
TC_2	nd	nd	3.67 ± 0.11	2.54 ± 0.07	nd
TC_3	nd	3.03 ± 0.09 b	nd	4.00 ± 0.12 a	3.26 ± 0.10 b
Cys-PC_2	nd	30.40 ± 0.59 a	31.02 ± 0.90 a	0.94 ± 0.07 b	0.71 ± 0.02 c
TC_4	nd	36.67 ± 0.78 a	36.50 ± 1.06 a	32.23 ± 1.25 b	6.77 ± 0.20 c
PC_3	nd	57.50 ± 1.09 a	52.30 ± 1.52 b	42.91 ± 1.72 c	4.80 ± 0.14d
TC_5	nd	nd	nd	59.12 ± 3.09 a	10.59 ± 0.31 b
desGly-PC_3	nd	45.11 ± 1.32 c	69.82 ± 2.04 b	105.96 ± 4.23 a	11.40 ± 0.33 d
Total NPTs	20.63 ± 1.04 d	196.76 ± 7.99 b	219.98 ± 11.11 b	274.00 ± 12.84 a	47. 62 ± 2.40 c

Table 4.7. Non-protein thiolic peptides (nmol GSH g^{-1} FW) in shoots of *S. atrocinerea* at 30 days of culture under different treatments. Differen letters for each row indicate significant differences among treatments on HSD test at $p < 0.05$. nd: not detected

Thiol	Treatment				
	Control	As III	As V	As V + P	As V + R
Cys	2.69 ± 0.08 c	1.99 ± 0.06 d	2.84 ± 0.10 c	4.38 ± 0.13 b	4.73 ± 0.13 a
GSH	32.10 ± 0.94 bc	30.41 ± 0.89 c	33.58 ± 0.98 b	27.57 ± 0.80 d	59.44 ± 1.73 a
PC_2	1.06 ± 0.03 d	3.02 ± 0.09 c	10.70 ± 0.31 a	7.03 ± 0.21 b	10.33 ± 0.30 a
TC_3	nd	1.02 ± 0.03 d	4.58 ± 0.13 a	2.78 ± 0.08 b	2.17 ± 0.06 c
PC_3	1.27 ± 0.04 e	1.39 ± 0.04 d	9.42 ± 0.27 a	5.54 ± 0.16 b	3.40 ± 0.10 c
desGly-PC_3	nd	nd	4.61 ± 0.13 a	3.62 ± 0.11 b	nd
Cys-PC_3	nd	nd	3.38 ± 0.10 a	2.02 ± 0.06 b	nd
PC_4	nd	nd	1.54 ± 0.04 a	0.91 ± 0.03 b	nd
PC_6	5.02 ± 0.15 a	3.32 ± 0.10 d	3.61 ± 0.11 c	3.50 ± 0.10 cd	4.30 ± 0.13 b
TC_6	5.10 ± 0.14 b	3.37 ± 0.13 d	4.78 ± 0.14 c	4.61 ± 0.13 c	5.78 ± 0.17 a
TC_7	1.47 ± 0.04 b	0.96 ± 0.03 d	1.00 ± 0.03 d	1.30 ± 0.04 c	1.96 ± 0.06 a
TC_8	6.31 ± 0.18 a	3.80 ± 0.11 c	1.83 ± 0.05 d	4.93 ± 0.14 b	5.22 ± 0.15 b
TC_9	6.15 ± 0.24 a	4.21 ± 0.12 d	2.83 ± 0.08 e	4.66 ± 0.14 c	4.99 ± 0.15 b
TC_{10}	0.95 ± 0.03 b	0.45 ± 0.01 d	0.55 ± 0.02 c	0.57 ± 0.02 c	1.71 ± 0.05 a
Total NPTs	62.13 ± 3.14 d	53.95 ± 2.72 e	85.24 ± 4.52 b	73.43 ± 3.75 c	104.02 ± 5.34 a

In shoots, the increase in NPTs in As-treated plants was not so large as in roots and the highest NPT synthesis was observed in As V + R treatment (**Table 4.7**). In control plants, Cys, GSH, PC$_2$, PC$_3$, PC$_6$ and five unidentified thiol compounds (TC$_{6-10}$) were detected (**Table 4.7**). Together with these thiols, *de novo* synthesis of desGly-PC$_3$, Cys-PC$_3$ and PC$_4$ was also observed in shoots of As V and As V + P plants (**Table 4.7**). Cys levels were higher in the arsenic treatments with bacterial inoculation and differences were observed for GSH concentrations among different treatments. In shoots of As V + R, the GSH concentration was almost twice the concentration observed in shoots of As V plants. By contrast, a slight decrease was observed for GSH levels in As V + P plants as compared to the As V plants. PC$_6$ content decreased under As exposure as did TC$_8$ and TC$_9$ (**Table 4.7**).

4.3.6   *Analysis of total flavonoids, condensed tannins and phenols*

In shoots, total flavonoid content remained unaltered in all arsenate treatments as compared to the control plants but not for As III-treated plants, where we observed a decrease. The analysis of condensed tannins showed the highest values in inoculated plants (As V + P and As V + R) and the same trend was observed for total phenols (**Fig. 4.4**).
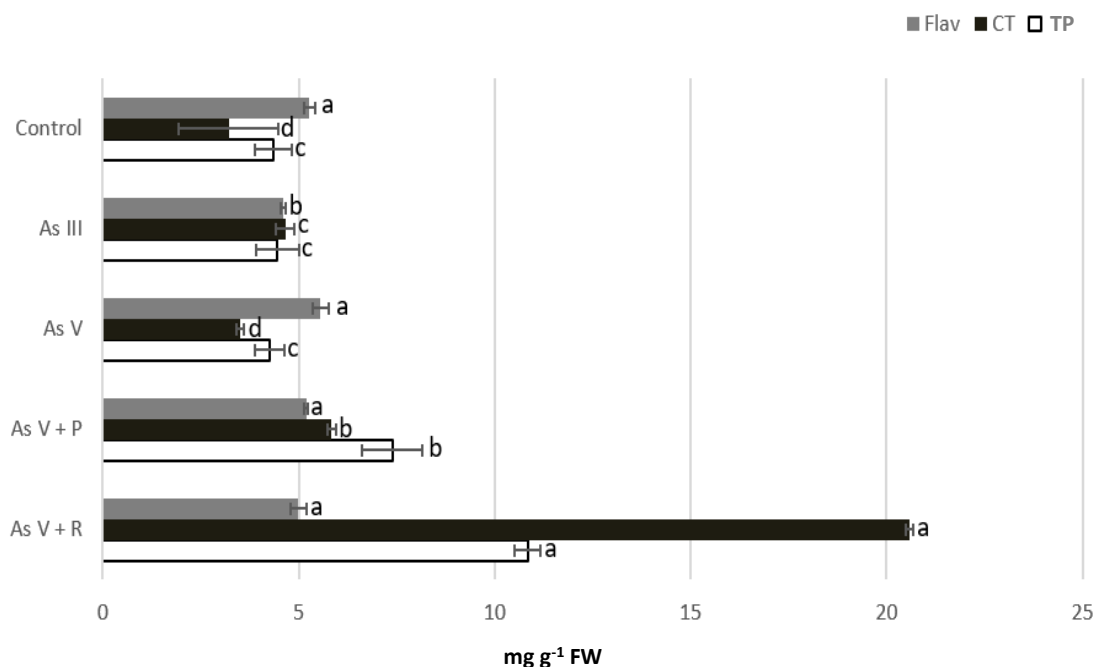
Fig. 4.4. Total flavonoids (Flav), condensed tannins (CT) and total phenols (TP) in shoots of *S. atrocinerea* at 30 days of culture under different treatments. Different letters for each compound indicate significant differences among treatments on HSD test at p < 0.05.
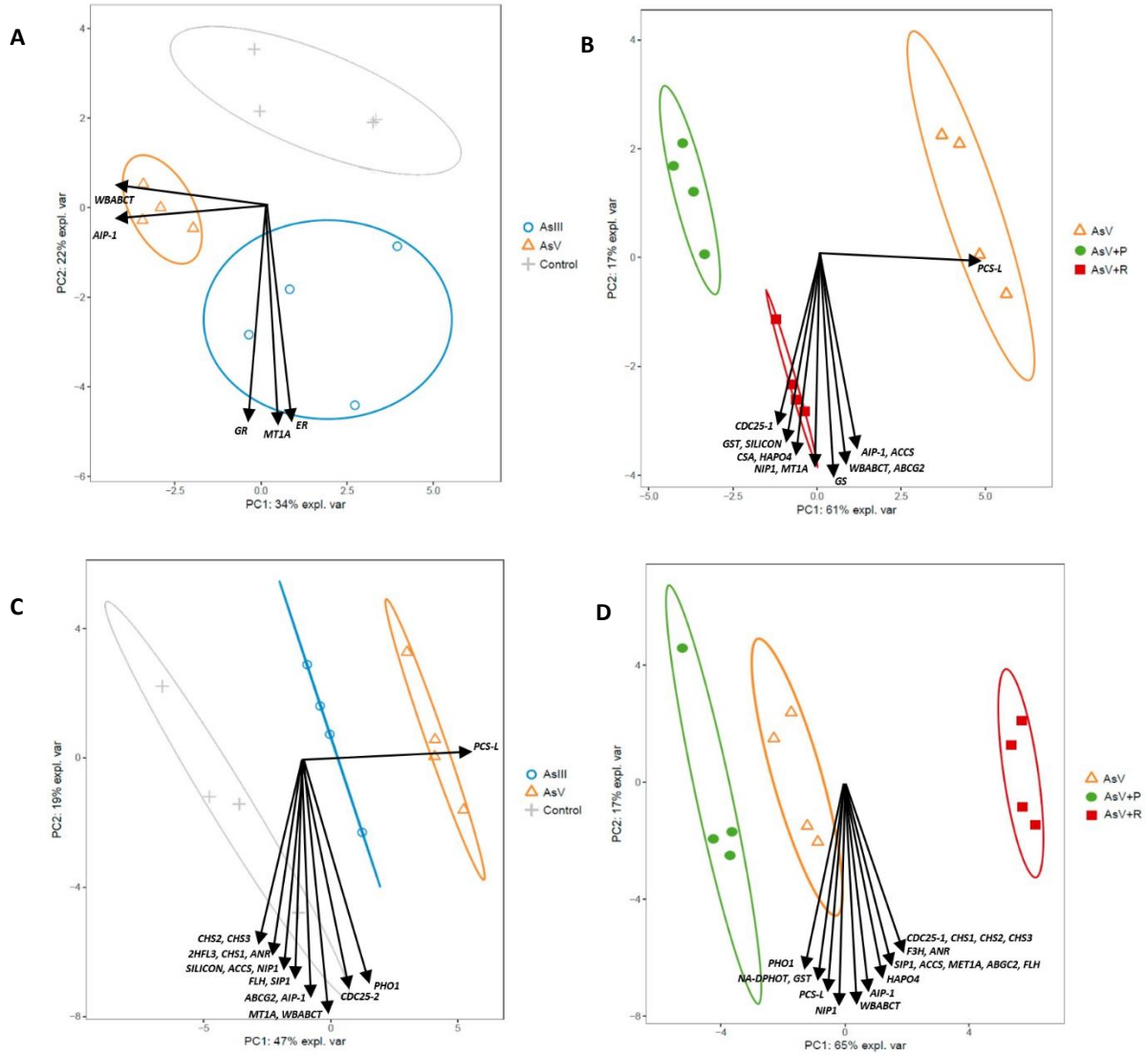
4.3.7 Gene expression

According to the PCA analysis (**Fig. 4.5**), samples from the same treatment clustered together indicating that the gene expression pattern was highly correlated among experimental conditions.

In roots, comparing plants grown in control conditions to those exposed to As, the principal component 1 (PC1) (34% variation) indicated a separation of samples from plants grown in the As III treatment from those of control or As V, which could be attributed to changes in transcript levels for the ethylene receptor protein (*ER*) and metallothionein (*MT1A*) (**Fig. 4.5A**). PC1 also indicated that under As V presence, samples grouped due to changes in transcripts for glutathione synthase (*GS*) (**Fig. 4.5A**). Principal component 2 (PC2) (22% variation) showed that samples of roots exposed to As and control conditions, differed by changes in transcript levels of an arsenite inducible protein (*AIP-1*) in As V and As III treatments, and that a regulation of transcripts of the white-brown-complex ABC transporter (*WBABCT*) is a characteristic of samples from As V treatment (**Fig. 4.5A**). When the additional effect of bacterial inoculations in roots were studied, PC1 (61 % variation) indicated that the presence of *Pantoea* sp. or *R. erythropolis* caused changes in the expressions of transcripts coding for arsenic-related transporters, such as a phosphate transporter (PHO1), a high-affinity phosphate transporter (HAPO4), a membrane silicon transporter (SILICON), an arsenate reductase (CDC25-2), AIP-1*, an aminocyclopropane-1-carboxylate synthase (ACCS), a glutathione transferase (GST), a phytochelatin synthase (PS-L), MT1A and WBABCT (**Fig. 4.5**B). In addition, *R. erythropolis* inoculation (As V + R) caused changes in transcripts for aquaporin (*NIP1*), a cellulose synthase (*CSA*), *GS*, and a vacuolar transporter (*ABCG2*) as compared to As V treatment.

Differences in gene expression between samples of shoots from plants exposed to As and control conditions were reflected, according to PC1 (47% variation), in changes of transcript levels related to the stress metabolism (*AIP-1* and *ACCS*) and the flavonoid pathway [chalcone synthases (*CHS1*, *CHS2* and *CHS3*), flavonoid 3'-hydroxylase (*FLH*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*2HFLR*) and anthocyanidin reductase (*ANR*)] (**Fig. 4.5C**). With respect to As speciation, As III treatment showed changes in transcripts coding for phosphate transporter PHO1, aquaporins NIP1, SIP1 and SILICON. There were also changes in transcripts coding for CDC25-2, MT1A and vacuolar transporters WBABCT and ABGC2 (**Fig. 4.5C**). Differences between inoculated and non-inoculated treatments in As V + P and As V + R samples (65% variation explained by PC1) were due to changes in shoot transcript levels of *HPO4*, *CDC25-1*, *AIP-1*, *ACCS* as well as flavonoid-related genes *CHS1*, *CHS2*, *CHS3*, *FLH*, *F3H*, *2HFLR*, anthocyanidin synthase (*ANS*)

and *ANR* (**Fig. 4.5D**). In addition, in As V + R treatment, there were changes in transcript levels of *PHO1*, a sodium-dependent phosphate transporter *NA-DPHOT*, *SIP1*, *NIP1*, thiol-related proteins *PCS-L* and *MT1A*, and vacuolar transporters *WBABCT* and *ABCG2* (**Fig. 4.5D**).

Fig. 4.5. Biplots of the principal component analysis (PCA) in samples of roots (**A, B**) and leaves (**C, D**) of *S. atrocinerea* exposed to arsenic at 30 days of culture under different treatments calculated with the normalized gene expression levels relative to the control plants (**A, C**) or As V plants non-inoculated (**B, D**) at each time point of at least four biological replicates, each containing at least one individual plant. For gene abbreviations see ***Supplementary Table 1.***

4.3.7.1   *Differential gene expression in roots*

Exposure to As, whether As III or As V caused an up-regulation of *AIP-1* of 5 and 2.7-fold, respectively (**Table 4.8**). However, the speciation state of arsenic also caused specific differences in gene expression pattern, as exposure to As III caused a 12-fold up-regulation of *ER* (**Table 4.8**) and 1.5 up-regulation of *MT1A*, whereas exposure to As V resulted in a 2-fold up-regulation of *GS* and 1.5 up-regulation of *WBABCT* (**Fig. 4.6A**).

Bacteria-inoculated treatments (As V + P, As V + R) caused a 1.4-fold down-regulation of *PHO1*. Likewise, *HAPO4* expression was 1.5 and 2-fold down-regulated in roots of As V + P and As V + R plants, respectively. Changes involving transcripts coding for arsenite transport-related proteins were also detected in inoculated treatments, such as *NIP1*, which was 2.4-fold down-regulated in As V + R plants, whereas there was a 1.4 and 4-fold down-regulation of *SILICON* in roots of As V + P and As V + R plants, respectively (**Fig. 4.6B**). Two transcripts of CDC25-like tyrosine phosphatase (an arsenate reductase), *CD25-1* and *CDC25-2*, were measured and there was a 2-fold down-regulation of the latter in roots of As V + R plants (**Fig. 4.6B**). In the case of thiol metabolism, we observed that the expression levels of *GS* were 1.7-fold up-regulated in As V + R plants, *GST* transcripts were 1.4 and 1.9-fold down-regulated in As V + P and As V + R plants, respectively, and a 1.8 and 2.6-fold down-regulation in As V + R plants were observed for phytochelatin synthetase (PCS) and PCS-L, respectively (**Fig. 4.6B**). *MT1A* transcripts were down-regulated in As V + P (2-fold) and As V + R (2.4-fold) plants (**Fig. 4.6B**). Changes in transcripts related to vacuolar accumulation of metals via PC-metal complexes were detected for *WBABCT* in roots of As V + P (1.5-fold up-regulation) and As V + R plants (16.4-fold up-regulation). In As V + R plants, there was also a 34.6-fold increase for *ABCG2* transporter transcripts (**Table 4.8**). With respect to arsenic stress related genes, there was an up-regulation of AIP-1 in roots of As V + P (2.2 fold) and As V + R plants (26-fold), as well as an up-regulation of *ACCS* under As V + P (8 fold) and As V + R treatments (911-fold). Transcripts of CSA were 1.9-fold down-regulated in roots of As V + R plants (**Table 4.8**).
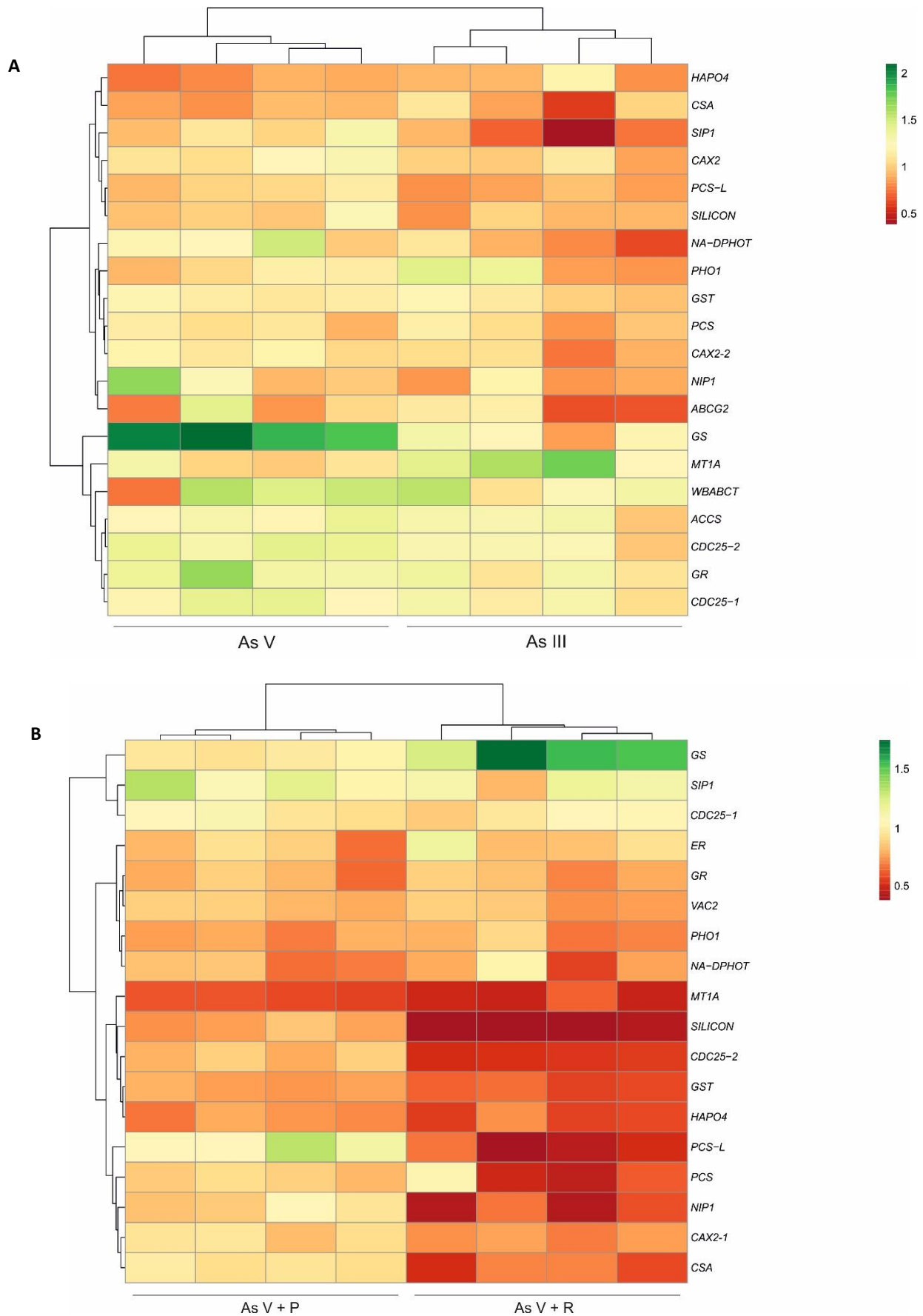
Fig. 4.6. Heat map representations of the gene expression data obtained in samples of roots of *S. atrocinerea* exposed to arsenic for 30 days and hierarchical clustering based on the most differentially expressed genes with a fold regulation equal or lower than 2 with respect to control **(A)** or non-inoculated As V plants **(B)**. Gene expression level values are the normalized expression relative to the non-exposed plants at each time point of at least four biological replicates, each containing at least one individual plant. Green-shaded rectangles indicate increased, while red-shaded rectangles indicate decreased gene expression. For gene abbreviations see ***Supplementary Table 1.***

4.3.7.2 Differential gene expression in shoots

The changes in gene expression in shoots of As-exposed plants from those under control conditions were apparent by an up-regulation in both As III and As V treatments of *AIP-1* (2 and 2.2-fold, respectively) and *ACCS* (3.7 and 2.1-fold, respectively), together with a down-regulation in the flavonoid pathway of *CHS1* (2.6 and 1.6-fold, respectively), *CHS2* (2.1 and 1.7-fold, respectively), *CHS3* (2.4 and 1.6-fold, respectively), *FLH* (1.5 and 1.5-fold, respectively), *2HFLR* (1.8 and 1.4-fold, respectively) and *ANR* (1.8 and 1.4-fold, respectively) (**Fig. 4.7A**, **Table 4.8**). In As III treatment, there was also an 1.4-fold up-regulation of *PHO1*, a down-regulation of aquaporins *SIP1* (1.5-fold), *NIP1* (1.4-fold) and *SILICON* (1.6-fold), a 1.5-fold up-regulation of *CDC25-2* and a 1.5-fold down-regulation of *MT1A* (**Fig. 4.7A**).

In As V treatment, changes in transcript levels could be observed when plants were inoculated. These included As-related transporters, with a 1.8 down-regulation of *PHO1* in shoots of As V + R plants, a 1.9 and 2.4-fold up-regulation in transcripts of *HAPO4* in As V + P and As V + R plants, respectively, a 2.4-fold down-regulation of *NA-DPHOT* as well as of *NIP2* in As V + R plants, and a 2.7-fold up-regulation of *SIP1* in As V + R plants (**Fig. 4.7B**, **Table 4.8**). Regarding As reduction, there was a 1.9 and 6.6-fold up-regulation of *CDC25-1* in As V + P and As V + R plants, respectively (**Table 4.8**). For genes related to the thiol metabolism, only changes were observed in As V + R plants, with a 1.7-fold down-regulation of *GST*, 2.5-fold down-regulation of *PCS-L* and a 3.9 up-regulation of *MT1-A* (**Fig. 4.7B**, **Table 4.8**). Genes related to vacuolar transport showed a 2.2 and 19.8-fold up-regulation of *WBABCT* and *ABCG2* in As V + R plants (**Table 4.8**). For stress-related genes, an up-regulation was observed in As V + P and As V +R plants of *AIP-1* (2.5 and 3.3-fold, respectively) and *ACCS* (3.9 and 7.9-fold) (**Table 4.8**). In As V + R plants, there was a 1.5-fold down-regulation of *CSA* gene expression (**Fig. 4.7B**). With regard to flavonoid pathway-related genes and opposite to As III and As V treatments, there was a transcriptional up-regulation in As V + P and As V + R plants of *CHS1* (2.6 and 7.5-fold, respectively), *CHS2* (4.0 and 15.3-fold), *CHS3* (2.7 and 10.1-fold), *F3H* (2.1 and 4-fold), *FLH* (2.1 and 3.2-fold), *2HFLR* (1.7-fold both), *ANS* (2.9 and 2.2-fold) and *ANR* (1.8 and 1.4-fold) (**Table 4.8**).
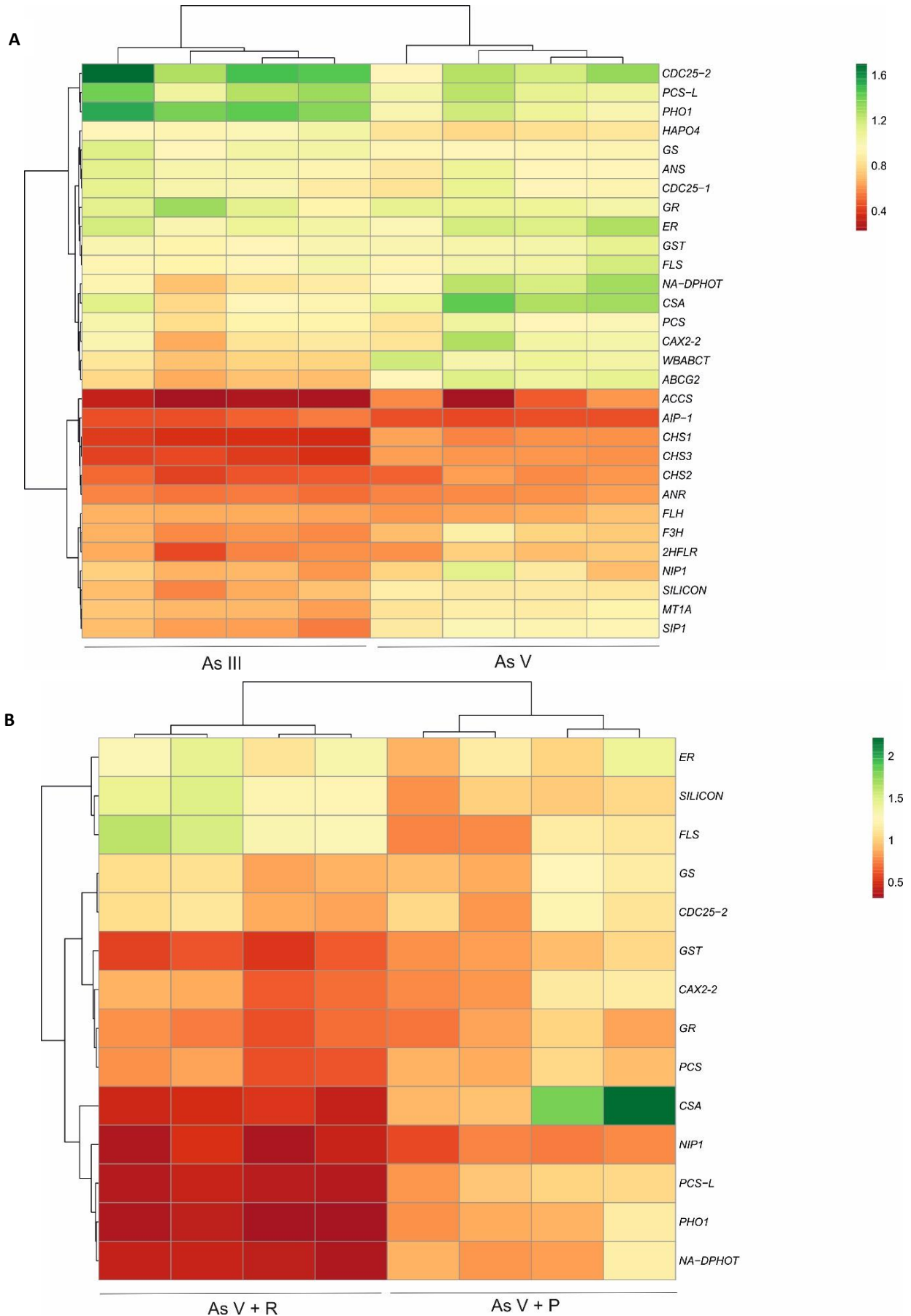
Fig. 4.7. Heat map representations of the gene expression data obtained in samples of leaves of *S. atrocinerea* exposed to arsenic for 30 days and hierarchical clustering based on the most differentially expressed genes with a fold regulation equal or lower than 2 with respect to control **(A)** or non-inoculated As V plants **(B)**. Gene expression level values are the normalized expression relative to the non-exposed plants at each time point of at least four biological replicates, each containing at least one individual plant. Green-shaded rectangles indicate increased, while red-shaded rectangles indicate decreased gene expression. For gene abbreviations see ***Supplementary Table 1.***

Table 4.8. Relative gene expression levels in roots and leaves of *S. atrocinerea* exposed to arsenic for 30 days of genes with a fold regulation higher than 2. Values are mean normalized expression relative control or As V plants (set at 1.00) ± S.D. of at least four biological replicates, each containing at least one individual plant. Statistically significant at $p < 0.05$. Different letters within each column and plant tissue indicate significant differences among time points on HSD test at $p < 0.05$.

Organ	Gene	Description	Fold change compared to control		Fold change compared to As V	
			As III	As V	As V + P	As V + R
Roots	*AIP-1*	Chaperone	5.05 ± 0.76 a	2.68 ± 0.30 b	2.23 ± 0.64 b	26.21 ± 3.81 a
	ER	Ethylene receptor	12.17 ± 5.66 a	1.43 ± 0.55		
	ACCS	ACC synthase			8.24 ± 3.91 b	911.03 ± 131.32 a
	WBABCT	Vacuolar transporter			1.51 ± 0.17 b	16.44 ± 4.66 a
	ABCG2				1.44 ± 0.55 b	34.57 ± 10.99 a
Leaves	*HAPO4*	High affinity P transporter			1.88 ± 0.18 b	2.35 ± 0.18 a
	SIP1	Aquaporin			1.38 ± 0.32 b	2.69 ± 0.33 a
	CDC25-1	Arsenate reductase			1.96 ± 0.34 b	6.59 ± 2.12 a
	AIP-1	Chaperone			2.51 ± 0.46	3.26 ± 1.82
	ACCS	ACC synthase			3.90 ± 1.35 b	7.87 ± 2.70 a
	MT1A	Metallothionein			1.01 ± 1.89	3.89 ± 0.43 a
	WBABCT	Vacuolar transporter			0.83 ± 0.28	2.16 ± 0.56 a
	ABCG2				1.15 ± 0.22 b	19.79 ± 9.62 a
	CHS1				2.58 ± 0.81 b	7.48 ± 2.86 a
	CHS2	Chalcone synthase			3.96 ± 0.80 b	15.28 ± 4.21 a
	CHS3				2.73 ± 0.78 b	10.09 ± 3.02 a
	FLH	Flavonoid 3'-hydroxylase			2.09 ± 0.60	3.21 ± 0.84
	F3H	Flavanone 3-hydroxylase			2.06 ± 0.33 b	3.98 ± 0.47 a
	ANS	Anthocyanidin synthase			2.87 ± 0.83	2.19 ± 0.33
	ANR	Anthocyanidin reductase			2.04 ± 0.65 b	3.64 ± 0.82 a

4.3.8   *Analysis of plant growth regulators (PGRs)*

Analyses of PGRs showed that in roots, auxin indole-3 acetic acid (IAA) was almost 7-fold higher in As V + R plants than under control conditions (**Table 4.9**). Cytokinin (CK) concentrations (trans-zeatin, tZ and trans-ribosylezatin, tRz) were higher in As III, As V + P and, especially, in As V + R plants, as compared to control plants, whereas plants of As V treatment showed the lowest CK concentration (**Table 4.9**). Gibberellins (GAs), especially GA9 accumulated at high concentrations in As V + R plants, whereas it decreased in As V + P plants as a consequence of a reduced GA1 and GA4 accumulation (**Table 4.9**). Brassinosteroid castasterone (BK) concentrations decreased in As V and As V + R plants. Salicylic acid (SA) showed its highest value in As V + R plants and its lowest in As V + P plants (**Table 4.9**). Abscisic acid (ABA) accumulated in roots of As V + R plants whereas jasmonic acid (JA) decreased in As V, As V + P and As V + R plants (**Table 4.9**).

In shoots, the IAA concentration increased in As V + R plants, as was similar for CKs [especially isopentenyl adenosine (IPA)] (**Table 4.10**). The latter was also seen in shoots of As V + P and As V plants (**Table 4.10**). Concentrations of BK decreased in plants under all As conditions (**Table 4.10**). SA accumulated in As V+ P plants and ABA had a 4-fold increase in As V + R plants, whereas it decreased in As III and As V + P plants (**Table 4.10**). No changes where observed for JA in plants of any of the treatments (**Table 4.10**).

Table 4.9. Hormone concentration (ng g^{-1} FW) in roots of *S. atrocinerea* at 30 days of culture under different treatments. Different letters for each plant tissue indicate significant differences among cultivation time points on HSD test at $p < 0.05$. nd: not detected.

| Hormone | Roots | | | | |
	Control	As III	As V	As V + P	As V + R
IAA	7.31 ± 1.40 b	5.01 ± 0.43 c	5.43 ± 0.62 bc	6.60 ± 1.27 bc	48.29 ± 1.34 a
tZ	2.74 ± 0.02 b	2.77 ± 0.20 b	2.95 ± 0.21 b	2.40 ± 0.05 c	6.27 ± 0.66 a
DHZ	0.49 ± 0.01 a	0.48 ± 0.06 a	0.54 ± 0.04 a	0.39 ± 0.01 c	0.46 ± 0.04 a
tRZ	nd	13.02 ± 1.48 b	nd	10.21 ± 2.34 b	77.93 ± 10.25 a
DHZR	2.33 ± 0.09 b	3.31 ± 0.88 a	3.20 ± 0.19 a	2.03 ± 0.15 c	2.96 ± 0.08 a
BA	1.07 ± 0.11 a	0.90 ± 0.05 a	0.58 ± 0.14 b	0.33 ± 0.02 c	0.44 ± 0.04 b
IP	0.52 ± 0.06 c	0.39 ± 0.03 d	0.24 ± 0.03 e	0.79 ± 0.02 b	1.34 ± 0.10 a
IPA	3.83 ± 0.39 a	2.21 ± 0.27 b	1.51 ± 0.10 c	3.16 ± 0.32 a	2.69 ± 0.20 b
GA1	12.92 ± 1.13 a	11.94 ± 0.83 ab	12.30 ± 0.55 a	10.83 ± 0.76 b	13.09 ± 0.78 a
GA3	0.78 ± 0.11 a	1.04 ± 0.03 a	1.37 ± 0.74 a	0.82 ± 0.17 a	1.29 ± 0.45 a
GA4	2.90 ± 0.26 a	0.55 ± 0.11 b	2.58 ± 0.04 a	0.72 ± 0.09 b	2.58 ± 0.22 a
GA9	3.60 ± 1.10 c	4.28 ± 1.19 b	2.60 ± 0.02 c	2.29 ± 0.30 c	3531.84 ± 62.18 a
GA20	nd	nd	nd	0.19 ± 0.06 a	0.27 ± 0.03 a
BK	150.20 ± 8.64 a	133.04 ± 8.63 a	76.69 ± 10.58 b	144.59 ± 10.59 a	67.20 ± 13.57 b
SA	125.37 ± 15.64 b	196.90 ± 6.03 c	144.96 ± 10.92 b	62.49 ± 5.49 d	498.57 ± 35.10 a
ABA	5.33 ± 0.82 b	5.68 ± 1.00 b	5.94 ± 0.40 b	6.85 ± 0.92 b	25.60 ± 2.99 a
JA	22.12 ± 1.24 a	18.37 ± 2.93 a	8.48 ± 1.79 b	12.05 ± 2.00 b	10.39 ± 1.07 b

Table 4.10. Hormone concentration (ng g^{-1} FW) in shoots of *S. atrocinerea* at 30 days of culture under different treatments. Different letters for each plant tissue indicate significant differences among cultivation time points on HSD test at p < 0.05. nd: not detected.

Hormone	Shoots				
	Control	As III	As V	As V + P	As V + R
IAA	13.41 ± 0.87 b	10.76 ± 1.94 b	12.94 ± 1.21 b	13.64 ± 1.15b	20.65 ± 2.44 a
tZ	3.20 ± 0.31 ab	2.78 ± 0.10 b	3.34 ± 0.20 a	2.47 ± 0.11 c	2.77 ± 0.08 b
DHZ	0.61 ± 0.03 a	0.48 ± 0.01 c	0.64 ± 0.07 a	0.52 ± 0.03 b	0.55 ± 0.02 ab
tRZ	7.35 ± 1.58 c	9.27 ± 1.64 bc	11.37 ± 0.63 ab	8.40 ± 0.97 c	11.23 ± 0.26 a
DHZR	2.62 ± 0.19 a	1.91 ± 0.13 b	2.93 ± 0.43 a	2.21 ± 0.19 b	2.01 ± 0.04 b
BA	1.86 ± 0.15 a	1.16 ± 0.09 b	1.45 ± 0.19 b	1.12 ± 0.10 b	0.72 ± 0.07 c
IP	0.14 ± 0.05 b	0.12 ± 0.04 b	0.18 ± 0.02 b	0.18 ± 0.03 b	0.41 ± 0.07 a
IPA	17.13 ± 1.64 c	18.23 ± 1.03 c	23.46 ± 1.05 b	25.28 ± 0.43 b	51.55 ± 1.25 a
GA1	13.38 ± 1.50 b	11.62 ± 0.95 bc	12.13 ± 0.66 bc	10.40 ± 0.65 c	20.34 ± 2.01 a
GA3	1.11 ± 0.15 a	0.76 ± 0.19 a	0.99 ± 0.08 a	1.07 ± 0.07 a	1.01 ± 0.23 a
GA4	1.67 ± 0.18 a	0.32 ± 0.01 e	0.55 ± 0.12 d	0.87 ± 0.15 c	1.32 ± 0.12 b
GA9	4.62 ± 1.94 b	2.71 ± 0.31 c	1.87 ± 0.18 d	3.33 ± 0.56 c	11.92 ± 1.12 a
GA20	3.87 ± 0.46 a	3.78 ± 0.92 a	4.31 ± 0.62 a	1.38 ± 0.09 b	nd
BK	790.40 ± 69.67 a	325.42 ± 50.79 b	345.18 ± 1.25 b	222.78 ± 2.61 c	68.26 ± 14.28 d
SA	66.05 ± 4.57 bc	57.38 ± 5.93 c	59.90 ± 3.85 c	83.48 ± 5.92 a	75.39 ± 7.54 ab
ABA	100.03 ± 9.14 b	80.82 ± 2.94 c	94.71 ± 7.05 b	76.31 ± 5.13 c	483.01 ± 0.66 a
JA	18.69 ± 1.81 a	12.43 ± 4.00 c	21.49 ± 8.26 a	17.78 ± 4.49 a	20.10 ± 3.71 a

4.4   DISCUSSION

Plant arsenic uptake relies on the As concentration available in the environment and, more importantly, on the As speciation in the soil or culture medium, with As III being more mobile than As V and readily available for the plant (Fitz and Wenzel, 2002; Martínez-Sánchez et al. 2011). Therefore, bacterial related arsenic speciation could influence plant As uptake. In paddy environments, it has been described that microbial reduction of As V to As III increases As mobility (Hu et al. 2015), resulting in greater As uptake and translocation in rice compared to other forms of As (Takahashi et al. 2004). Nevertheless, different As speciation can also lead to alterations in toxicity for both microorganisms and plants. For most bacteria, As V reduction to As III followed by As III efflux helps As detoxification, and in some cases serves as an energy source (Hamamura et al., 2014); the bacteria that are efficient in reducing As V are more resistant to this form (Ghosh et al., 2015), as happens with *Pantoea* sp. and *R. erythropolis*, used in our work, both highly resistant to As [*Pantoea* sp. up to 50 mM As V and 20 mM As III, and *R. erythropolis* up to 100 mM As V and 20 mM As III, in 284 liquid medium]. When used in inoculation experiments, both bacteria were able to grow in the culture media with 18 mg L^{-1} As V and were able to colonize *S. atrocinerea* root and leaf tissues (**Fig. 4.1**) After reduction to As III, it can be easily taken up by plants and cause undesired effects since it is more phytotoxic than As V and, as a consequence, it is not accumulated by the plant (Abedin et al., 2002; Quaghebeur and Rengel, 2003; Hokura et al., 2006; De la Rosa et al., 2006; Cordos et al., 2006).

Under As V treatment, the 2% As III observed at the end of the experiment (**Fig. 4.3B**) could be attributed to its reduction and the subsequent arsenite efflux as the plant detoxification mechanism (Xu et al., 2007; Park et al., 2016). The further reduction up to 10% and 93% of As V to As III that was respectively observed in As V + P and As V + R treatments can be attributed only to the bacterial metabolism. The arsenate detected in As III treatment on the other hand (**Fig. 4.3B**) can be attributed to plant metabolism as seen in other studies (Huang et al., 2011), since there was no As V speciation detected in the As III solution without plants (data not shown). At the end of the exposure, higher pH values were detected in As III and As + R treatments, where arsenite concentration in the medium was the highest (**Fig. 4.3B**); the lowest pH value was registered for As V + P and As V treatments. As suggested by Park et al. (2016), protons excreted to the media by plant roots may contribute to the reduction of As V to As III, a process that consumes H$^+$ and thus, as observed in that experiment, increases the pH. As a consequence, we observed a greater As accumulation in As V + P plants (**Table 4.3**), which could be explained by (1) a higher As availability due to a moderate As V reduction to As III and/or (2) an increased root biomass

attributed to bacterial inoculation (Sessitsch et al., 2013). On the contrary, the As reduction activity of *R. erythropolis* caused a higher As III production in the medium and this caused a decreased As uptake by the plant as a consequence of an increased As phytotoxicity. It also needs to be taken into account that As can also be retained in the bacterial biomass (Ghosh et al., 2011). After 30 days of As exposure, the amount of As in the medium of As V treatment decreased to similar concentrations as in the As V + R treatment, and the lowest As concentration was detected in the As V + P treatment (**Fig. 4.3A**). In relation to the plant As accumulation, a decrease of As in the medium in the As V + P treatment probably is a result of a combination of As plant uptake and As accumulation into bacterial biomass. In the As V + R treatment however, the decrease of As in the medium most likely is a reflection of its accumulation into bacterial biomass, as As accumulation in roots was low, which is probably a consequence of the higher As III phytotoxicity.

Most of the As speciation experiments described in literature propose As V as being the predominant As form in leaves of As-exposed plants (Kertulis et al. 2005; Zhang et al., 2009; Yan et al., 2012; Park et al., 2016). However, in our study, As mainly accumulated in the form of As V in shoots of *S. atrocinerea* in all treatments assayed (**Fig. 4.3C**, **Table 4.3**), as was also reported in some other plants (Bohari et al., 2002; Tlustoš et al., 2002). The As V + R plants showed less As V in roots than those inoculated with *Pantoea* sp., although the total As concentrations in shoots was similar. So it seems that *Pantoea* sp. inoculation prevents As translocation in *S. atrocinerea*, despite an increased As concentration in roots (**Table 4.3**). This result is in agreement with the observations of Singh et al. (2016) and Thongnok et al. (2018) where bacterial inoculation to rice reduced the As translocation in shoots, especially in edible parts, while maximum As accumulation was observed in inoculated roots. This is possibly attributed to the production of inorganic sulfide and thiol-rich compounds induced in rice by bacteria (Thongnok et al., 2018) and/or to an enhanced vacuolar sequestration in roots. On the other hand, the concentration of As III in roots of As V and As V + P plants indicate that there is an efficient reduction of As V in the root cells, whereas a reverse scenario was observed in roots of As III and As V + R plants, which showed the oxidized As V as the predominant form (**Fig. 4.3C**, **Table 4.3**). According to the studies of Pickering et al. (2006) on *Pteris vittata*, As will be transported from roots to shoots in the As V oxidized form. Nevertheless, As V translocation will be limited, as most of the As is reduced in the roots to As III, which forms complexes with PCs and is sequestered to the vacuole (Zhao et al., 2009). The latter also coincides with lower NPT concentrations in roots of As V + R plants as compared to As V+ P plants (**Table 4.7**), which will be discussed later on.

As roots are the first organ coming into contact with the cultivation medium and since the translocation rate was very low in all the treatments, accumulation of As was always higher in roots than in shoots. Nevertheless, the higher As accumulation in roots of *S. atrocinerea* in As V + P plants does not correlate with an increased P content as it has been reported in other bacterial inoculation studies (Lou et al., 2010). For the As III and As V treatments, our results agree with those obtained by De la Rosa et al. (2006), where the As treatments reduced P concentrations in roots, although only exposure to As V reduced the P concentration in leaves. As V uptake occurs via the phosphate system, even replacing P in the phosphate groups of biomolecules (Wang et al., 2002; Patra et al., 2004). Therefore, in the case of As V, it is very likely that the metalloid entered the plants via phosphate uptake, thus reducing P concentrations in roots and its subsequent transport to the leaves. However, *in vitro* inoculation of the As-hyperaccumulating *P. vittata* with As-reducing bacteria promoted the formation of reduced As III in the culture medium, which helped plant P uptake (Han et al., 2016). In addition to the differential experimental setup and plant species used, a trade off in the As speciation between As V, limiting P uptake, and As III, being more phytotoxic, might explain this opposite result (Tu and Ma, 2003). In this way, since phytotoxicity symptoms were only observed in As V + R plants, this might explain the reduced P uptake in these plants. On the other hand, the predominant As V speciation in the medium of As V and As V + P conditions might relate to P uptake competition. Further data to explain the role that the bacterial inoculation can play in As-P interaction in willow, is the observed overall downregulation of phosphate channels transcripts, i.e. *PHO1* and *HAPO4* in both inoculated treatments (**Fig. 4.6B**) and additional research is needed to unravel the As-P interactions in willow and to relate it with the plant's metabolism.

It has been reported that the precise position, morphology and extent of roots can influence contaminant uptake (Remans et al., 2012). Furthermore, root system architecture is a major factor that is highly adaptive to increase tolerance to abiotic stresses such as nutrient deficiency, drought and salinity (Hinsinger et al., 2011). Inoculation of plants with *Pantoea sp.* (As V + P) resulted in an increased root biomass (**Fig. 4.2C**), which is in agreement with other inoculation studies (Nie et al., 2015). An increased root biomass in As V + P plants in the present study can be attributed to plant-growth-promoting (PGP) inoculant traits as a response to As-induced P deficiency, which is accentuated by the bacterial metabolic activity under As presence.

Concerning the micronutrients, the Fe uptake and translocation were more affected than other elements upon As exposure (**Table 4.2**). Arsenic and Fe were mainly concentrated in the roots, assuming an interaction of these two elements as was also previously observed in rice seedlings (Shaibur et al., 2008). One of the symptoms of As

toxicity in roots is the formation of Fe-plaques at the root surface (Shaibur et al., 2008; Shaibur et al., 2013). Both *Pantoea* sp. and *R. erythropolis* proved positive *in vitro* for siderophore production (**Fig. 4.1**) and siderophores are effective in providing Fe to the plant (Radzki et al., 2013). Nevertheless, the Fe might be trapped in Fe-plaques at the root surface level and as such limit translocation to the shoots of As V + P and As V + R plants, where bacterial inoculation resulted in decreased shoot Fe concentrations (**Table 4.2**).

It has been described that an increased supply of Zn to As-exposed plants augments their ability to withstand As toxicity (Srivastava and Shrivastava, 2017). In our data, we observed that Zn translocation was higher in As V and As V + P plants, whereas Zn strongly accumulated in roots of As V + R plants. This correlates with our findings on phytotoxic responses, because Zn is an esential micronutrient, which regulates abiotic stress responses and mitigates arsenic toxicity by modulating ROS and antioxidant function (Das et al., 2016)).

In order to prevent cellular toxic effects, chelation and sequestration are important mechanisms to detoxify free As. In *S. atrocinerea* roots there is a correlation between the concentrations of As and PCs (**Table 4.6** and **Table 4.7**). Since PCs bind arsenic better than GSH due to the formation of more stable complexes (Matsumoto et al. 1990), it was not unexpected that PC_2 and PC_3 contents were higher in those treatments with a higher As accumulation in roots (As V and As V + P), and that a similar trend was observed for desGly-PC_2 and desGly-PC_3, especially in As V + P plants. The high total NPT concentrations observed in roots under As V + P treatment, together with an up-regulation of *WBABCT* transporter transcripts (**Table 4.8**) can provide an effective mechanism to reduce As toxicity by its PC-complexation, sequestration into the vacuoles and prevention of its translocation to the leaves (Mishra et al., 2008). As NPTs decreased in roots of As V + R plants (**Table 4.6**), cysteine (Cys) concentrations, the main sulfur donor in the biosynthesis of sulfur-rich compounds (Na and Salt, 2011; Zagorchev et al., 2013), increased. Nevertheless, *de novo* synthesis of TC_5 appeared in roots of inoculated plants. This could indicate that the presence of this thiolic compound is related to bacterial inoculation, as was previously observed in rice by Thongnok et al. (2018).

In shoots, As detoxification can be related to *de novo* synthesis of PC_3 in As V, As V + P and As V + R plants, as well as desgly-PC_3, Cys-PC_3 and PC_4 in As V and As V + P plants. In contrast to the roots, the highest total NPT concentrations were observed in shoots of As V + R plants. Also in these plants, the As III concentration, specific for thiol complexation (Tripathi et al., 2012), was the highest (**Table 4.7**). Once inside the leaves, As V can readily be reduced to As III by *R. erythropolis*, which is reflected by the up-regulation of *CDC25- 1* (arsenate reductase) in leaves of As V + R plants (**Table 4.8**).

It has been reported that the photosynthetic pigments are susceptible to As (Azizur Rahman et al., 2007; Dutta and Mondal, 2014) through the inhibition of tetrapyrrole biosynthesis (Mishra et al., 2014, 2016). Furthermore, it has been suggested that As can hinder chlorophyll formation by decreasing the Fe concentration in shoots (Shaibur et al., 2008), a phenomenon observed in all As-exposed plants in our study (**Table 4.4**). Arsenate is described as the most harmful form for the photosynthetic pigments (Carbonell-Barrachina et al., 1998; Srivastava and Shrivastava, 2017), which coincides in our case with decreased concentrations of chlorophyll *a* and *b* as well as carotenoids in the treatments with the lowest Fe content (As III, As + P, and As V + R). Anthocyanins are non-enzymatic antioxidants that generally accumulate during As exposure (Finnegan and Chen, 2012) as was also observed in our study (**Table 4.4**). The antioxidative properties of anthocyanins arise from their high reactivity as hydrogen or electron donors, from the ability of the polyphenol-derived radicals to stabilize and delocalize the unpaired electron, and from their ability to chelate transition metal ions (Duan et al., 2007). As previously proposed, our results support the fact that, together with a decreased P uptake, anthocyanin synthesis can be considered as a visible marker of As stress (Catarecha et al. 2007).

In hyperaccumulator species, arsenic has been reported to increase enzymatic and non-enzymatic antioxidant mechanisms (Srivastava et al. 2005; Singh et al., 2006), whereas in non-hyperaccumulators, arsenic induces an oxidative stress in terms of enhanced lipid peroxidation, H_2O_2 accumulation and up-regulation of several scavenging enzymes (Hartley-Whitaker et al., 2001a). However, we observed that As-induced stress in roots of *S. atrocinerea* is not related to enhanced lipid peroxidation or H_2O_2 accumulation (**Table 4.5**), as was also seen in other studies (Singh et al., 2007; Czech et al., 2008). In shoots, however, H_2O_2 accumulation was increased in As III, As V + P and As V + R plants, whereas lipid peroxidation increased only in As V + R plants (**Table 4.5**). This clearly reflects the As toxicity linked to its speciation, and shows that no apparent membrane damage was observed unless high As III concentrations were present. Proline is a well-known osmoprotectant that highly accumulates in plants under As stress (Mohd et al., 2017; Abbas et al., 2018). When willow was inoculated with *R. erythropolis*, phytotoxic symptoms were noticed in As V + R plants, which correlates to a doubling of the proline concentration in the shoots as compared to control plants, which was not apparent in As V + P plants (**Table 4.5**).

Synthesis of phytohormones is strongly affected after exposure to heavy metals (Maksymiec, 2011). When willow was exposed to As V, we observe a reduction in CK concentrations in roots that was also reported by Mohan et al. (2016), who observed an increase of As V reductase activity and PC and GSH accumulation in *Arabidopsis* plants in response to As V. Also modulation of BK and JA was observed as previously reported in

Brassica (Kanwar et al., 2015, Farooq et al., 2016). In general, the role of PGRs in plant development is well described, however, their role in response to abiotic stress is less well understood. This becomes even more complicated in inoculation experiments since endophytic as well as rhizosphere bacteria can also synthesize plant hormones (Glick, 2012). From our results, it seems that bacterial inoculation plays a role in PGR synthesis as reflected by the opposed responses of SA and GAs in roots (**Table 4.9**) or ABA in shoots (**Table 4.10**) between non-inoculated and inoculated plants. Moreover, in As V +R plants presenting As-induced toxicity symptoms, we can see major changes of ABA, IAA, CKs, GAs in both roots and shoots, previously indicated as important regulatory factors in plant adaptation to As stress (Srivastava et al., 2015; Krishnamurthy and Rathinasabapathi, 2013; Mohan et al., 2016). Nevertheless, taking all data together, it is clear that phytohormones act in the integration of growth control and stress response, but their specific role in plant responses to arsenic requires further investigation.

The transcriptional changes induced upon exposure to non-essential toxic metals are part of a transcriptional response to preserve cellular homeostasis (Herbette et al., 2006; Mendoza-Cózatl et al., 2011) and these changes were studied in *S. atrocinerea* in the presence of arsenic and bacteria. As previously shown, only As V + R plants suffered from As phytotoxicity, which is in agreement with a down-regulation of *CSA* (**Fig. 4.6B**), an important gene in cellulose biosynthesis and hence plant growth, that is translated into a lower shoot biomass of As V + R plants (**Fig. 4.2C**). Moreover, we observed a great conversion of As V to the more phytotoxic As III in the As V + R culture medium (**Fig. 4.3C**). Therefore, differences in transcripts coding for arsenite transporters in *S. atrocinerea* roots could be expected. The transcriptional down-regulation of the aquaporin *NIP1* (**Fig. 4.7**B) that has been reported as responsible for arsenite uptake into the roots (Ma et al. 2008), can be interpreted as a way to limit As uptake and toxicity. Similarly, the down-regulation of *SILICON* transcripts, previously identified as the major entry point of arsenite and methylated arsenite in the roots, in As V + P and As V + R plants can be explained in this way (Li et al. 2009). The main mechanisms for As V reduction are arsenate reductases for which GSH acts as an electron donor (Dhankher et al. 2002). In *S. atrocinerea*, CDC25-like tyrosine phosphatases are involved in arsenate reduction to arsenite. Nevertheless, differential gene expression profiles, such as the upregulation of *CDC25-1* and downregulation of *CDC25-2* in both shoots of As V + P and As V + R plants (**Table 4.8** and **Fig. 4.7B**), should be further explored to clearly unravel the role of CDC25-like tyrosine phosphatases in arsenic speciation.

As mentioned before, GSH, PCs and other NPTs are important metabolites in As detoxification and the *GS* up-regulation in As-exposed *S. atrocinerea* (**Fig. 4.6**) confirms the

importance of GSH in As-tolerance (Alcántara-Martínez et al., 2018). Nonetheless, despite a clear up-regulation of *GS* in roots, GSH concentrations in roots of As-treated plants were lower than in control plants (**Table 4.6**). Therefore, the *GS* up-regulation is probably a response to the need of a great GSH turnover due to its use in PC production or in the non-enzymatic As V reduction, which coincides with the fact that the highest level of *GS* transcripts corresponds to As V and As V + R plants, the treatments with the lowest GSH concentration (**Table 4.6**). Although PCs were synthesized under arsenic exposure and their concentration increased over time, there were no changes in transcripts coding for PCS or PCS-L, with the exception of a decrease in roots and leaves of As V + R plants (**Fig. 4.6B**). As suggested by Cobbett (2000), *PCS* induction is unlikely to play a significant role in regulating PC biosynthesis. These enzymes are constitutively expressed at relatively high levels and are generally unaffected at the transcription level after exposure of cell cultures or plants to heavy metal(loid)s (Rea et al., 2004).

After complexation into As-PC complexes, arsenic should be transported via ABC transporters and stored into the vacuole. The ATP-binding cassette (ABC) transporters in plants are potentially involved in a number of plant processes (Campbell et al., 2003), and a role in vacuolar As sequestration and other metal(loid)s via PC complexation has been reported (Song et al., 2010; Park et al., 2012). Furthermore, these ABC proteins are likely to transport a very broad range of substrates that can be involved in the pathogen response and in the transport of chemicals that mediate pathogen resistance (Kim et al., 2007). Only high up-regulations of *ABGC2* and *WBABCT* transcripts were detected in roots and shoots of As V + R plants (**Table 4.8**). This indicates the importance of these transporters in plant As responses and constitutes an interesting target gene to modulate As accumulation and transport during phytoremediation processes.

Regarding arsenic stress-related genes, 1-aminocyclopropane-1-carboxylic (ACC) acid is the direct precursor in the biosynthesis of the stress hormone ethylene (Van de Poel and Van Der Straeten 2014). Although no changes in root *ER* transcripts were detected in *S. atrocinerea*, *ACCS* was up-regulated in those conditions where As III concentrations in the medium were higher and/or in the presence of bacteria (As III, As V + P and As V + R). Consistent with the phytotoxic responses, *ACCS* transcripts were the most up-regulated in As V + R plants. The cysteine- and histidine-rich RNA-associated protein, AIP-1, is a highly conserved gene selectively activated by arsenite in many cell types and organisms. In *Caenorhabditis elegans*, the inactivation of the aip-1 homolog compromises the survival of worms exposed to arsenite, but not to other stressors (Sok et al., 2001). In *S. atrocinerea*, *AIP-1* transcripts showed the highest up-regulation upon As exposure among the different genes analyzed, but this response was only observed in roots (**Table 4.8**). In *S. atrocinerea*

shoots, however, *AIP-1* up-regulation was restricted to bacterial-inoculated plants, whereas a down-regulation was detected in the non-inoculated As III and As V plants. These results suggest that AIP-1 in shoots is not induced by As stress but by the bacterial inoculation. It is known that bacteria can hijack the plant stress chaperone machinery in leaves during penetration (e.g. virulence or colonization) (Jelenska et al., 2010). In our experimental setup, the bacteria were able to colonize shoot tissue (**Fig. 4.1**) so we can speculate that they might interact with the chaperones and increase *AIP-1* expression.

It has been hypothesized that flavonoids can have a role in metal chelation (Winkel-Shirley, 2002). Davis et al. (2001) also proposed tannins as metal-binding compounds in hyperaccumulators, which would establish a resemblance in metal stress tolerance mechanisms between hyperaccumulators and *S. atrocinerea*. Although our previous studies (**Chapter 3**) suggest a potential role for tannins as an early response in As-induced oxidative stress mitigation in *S. atrocinerea*, the gene expression results at 30 days culture showed no indication that *S. atrocinerea* uses the phenylpropanoid pathway to cope with As toxicity, as reflected in the concentration of total flavonoids, condensed tannins and total phenols (**Fig. 4.4**). However, an up-regulation of transcripts related to anthocyanins or flavonoids was seen in the bacterial-inoculated treatments (**Table 4.8)**, which also coincided with an increase in condensed tannins and total phenols in these conditions when compared to As V plants, (**Figure 3**). This is not surprising, since flavonoids have been shown as crucial signaling molecules in the rhizobia nitrogen-fixing symbiosis (Liu and Murray, 2016) and a protective role of anthocyanin-containing cells against damage by infection-related ROS has been reported (Kangatharalingam et al., 2002). In addition, this high level of up-regulation of genes toward tannin biosynthesis under bacterial inoculation is interesting in the context of recent research published by Gonzalez et al. (2015) that suggested cross-tolerance of pollutant-treated *Salix* against arthropod herbivory biotic stress, where by increasing leaf tannin concentrations it confers a plant advantage by reducing arthropod predation in field conditions. In general, plant phenolics play an important role in defense against numerous environmental stressors, including excessive concentrations of toxic metal (Wozniak et al. 2017) and it has been discussed that rhizosphere and endosphere microbial communities impact phenolic signaling (Mandal et al., 2010), probably due in our case, to the discussed bacterial As transformation and differential As plant accumulation.

4.5 CONCLUSIONS

Our *in vitro* experiments showed that soil bacteria *Pantoea* sp. and *Rhodococcus erythropolis* associated to *Salix atrocinerea* can cause changes in As speciation and influence its availability. The inoculation with the bacterium *Pantoea* sp. increased the As phytostabilization, whereas inoculation with *R. erythropolis* stimulated the reduction of As V to As III in the medium, making it more mobile and available for plant uptake, which is an advantageous characteristics for their potential use for *in situ* field phytoremediation. Nevertheless, as As III is more phytotoxic and as such may reduce the host plant's growth and hence biomass production, this should be further explored under field conditions.

Interestingly, both bacteria limited As accumulation in *Salix* shoots due to different metabolic processes. Transcripts for key transporters mediating metal and arsenic uptake, tolerance, distribution and vacuolar accumulation in *S. atrocinerea* behaved in remarkable different ways in the presence or absence of As/bacteria. Our results provided experimental verification of the significance of metabolic flux through the thiol synthesis pathway, together with As and As-thiol vacuolar transporters to manipulate tolerance, accumulation and allocation of As within the plant.

The potential of the As-tolerant and As V-reducing bacteria to function synergistically and modify arsenic toxicity, mobility and accumulation in willow plants, unequivocally illustrates the need to elucidate the complex metabolic interactions between plants and their associated rhizosphere/endosphere microbiomes in field conditions, in order to design effective approaches for As-polluted soil phytoremediation.

Chapter 5

Bacterial Bioaugmentation causes differential Trace Element Accumulation and modulates Detoxification Mechanisms in *Salix atrocinerea* growing in a Multi-Polluted Soil

5 Bacterial Bioaugmentation causes differential Trace Element Accumulation and modulates Detoxification Mechanisms in *Salix atrocinerea* growing in a Multi-Polluted Soil

5.1 INTRODUCTION

Anthropogenic activities such as industrial processes, have increased the release of toxic concentrations of hazardous substances into the environment, threatening human health and other organisms. Trace elements (TEs) identified in the polluted environments include arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb) and zinc (Zn) (Lone et al., 2008), and are a major environmental problem (Tchounwou et al., 2012), because soils become unsuitable for cultivation and for industrial activities, and therefore they are abandoned indefinitely (Tóth et al., 2016b). Many kind of processes lead to dispersion of TEs, such as As, which can pollute the drinking water supply and surrounding land with undesirable impacts on the food chain and animal health (Kaur et al., 2011; Gallego et al., 2016). To eliminate these TEs from soils, remediation techniques based on biological processes offer alternatives to the most conventional civil engineering methods (Vangronsveld et al., 2009; Kidd et al., 2015). Bioremediation is the use of living microorganisms for the cleanup of environmental toxic pollutants (Ayangbenro and Babalola 2017; Mesa et al., 2017). Since many microorganisms possess the ability to degrade, transform, or chelate various toxic chemicals in the environment, they provide strategies to combat environmental pollution (Dzionek et al., 2016). A related technology is phytoremediation, defined as the use of plants to eliminate pollutants from the soil to reduce their harmful effects (Salt et al., 1998). Plants have the necessary genetic, biochemical, and physiological requirements to establish themselves in polluted areas where they thrive at high TE concentrations, excluding or accumulating these elements in their tissues (Gajić et al., 2018). Phytoextraction is based on the use of plants that take up TEs from soil and translocate them to aboveground tissues, where they accumulate (Lombi et al., 2008), whereas phytostabilization refers to plants that accumulate TEs mainly in their roots and can be employed to avoid the spread of pollutants by weathering processes (Vangronsveld et al., 2009).

Excess TE accumulation generates stress in plant cells because it alters the cellular redox homeostasis and increases the amount of reactive oxygen species (ROS) that must be

eliminated by the antioxidative defense systems (Birben et al., 2012) such as ROS quenching enzymes and the synthesis of compounds like glutathione (GSH), proline, carotenoids and phenolics (Abbas et al., 2018). The toxic effects of TE accumulation inside the plant can be also diminished by binding them to chelating compounds (Hartley-Whitaker et al., 2001b; Batista et al., 2014), and then storing the complexes into the vacuoles (Song et al., 2010). Among the TE-binding ligands in plant cells, phytochelatins (PCs) and metallothioneins (MTs) are the best characterized (Cobbett and Goldsbrough, 2002). PCs are a family of non-protein thiols that act as chelators and are made of increasing repetitions of the dipeptide ɣ-glutamyl-cysteine with glycine as the terminal amino acid $[(\gamma\text{-Glu-Cys})_n\text{-Gly}]$ where n = 2-11, binding the pollutants through a "-SH" group from cysteine (Hirata et al., 2005). PCs have been identified in a wide range of plants, fungi and microorganisms and they are synthesized from GSH by the enzyme phytochelatin synthase (PCS), which activation requires the presence of TEs, Cd being the most effective inducer (Clemens, 2006). Interestingly, PC molecules not only have a high TE-binding capacity but also strong scavenging activity against ROS (Tsuji et al., 2002). MTs which are also important metal chelators in plant cells, in contrast to PCs, are encoded by genes and thus are products of mRNA translation (Anjum et al., 2015).

Since microbial processes play a major role in TE-cycling, effective phytoremediation of polluted soils involves interactions with the specific plant-associated microbes. The mechanisms of TE uptake, accumulation, exclusion, translocation, osmoregulation and compartmentalization vary with each plant species and determine its suitability for phytoremediation (Powell et al., 2002). This also varies in function of its associated microorganisms (Furini et al., 2015). It is known that microbial processes or metabolites produced by soil or rhizosphere bacteria change the TE redox state and thus solubility and availability for its uptake by the plant (Kuffner et al., 2010). Besides, bacteria can produce siderophores, auxins, ACC-deaminase or solubilize phosphate. All this can improve plant growth in polluted soils and can accelerate the removal of the pollutants during the phytoremediation process (Luo et al., 2011). Indeed, an increase of plant biomass can be translated into a higher efficiency of phytoextraction (Weyens et al., 2009c).

The long time required to decrease TE contents in soils prevents the use of phytoremediation alone on an industrial scale, a drawback that can be mitigated with biotechnological approaches involving high-biomass, fast-growing crops (Vangronsveld et al., 2009; Pidlisnyuk et al., 2014; Jiang et al., 2015). The use of woody plants in phytoremediation implies a greater biomass production that can be used as biofuel or in lumber industry with subsequent economical profit (Witters et al., 2009, 2012 a,b; Rockwood et al., 2004). In addition, phytoremediation with woody species, such as willow,

shows various advantages compared to the use of herbaceous plants (Meers et al., 2007; Thijs et al., 2018), for example, a more extended root system that can reach a bigger soil volume and therefore limit markedly the entry of pollutants into the environment (Mench et al., 2009).

The subsequent potential optimization step is to focus on soil microorganisms which could accelerate this depollution process (Janssen et al., 2015; Mesa et al., 2017), and, in fact, the better understanding of the interactions between microbes and the plant genetic functions that was achieved in the last decade has contributed to improve the efficiency of environmental clean-up (Ayangbenro et al., 2017, Upadhyay et al., 2018). One of the potentially useful approaches derived from that knowledge, is the implementation of phytoremediation techniques in combination with bioaugmentation, defined as the introduction of specific competent strains or consortia of microorganisms, in order to enhance pollutant degradation or removal from soil (Mrozik and Piotrowska-Seget, 2010).

The debate over the efficacy of delivering suitable microorganism exists for a long time (Vogel, 1996), mainly due to the difficulty in predicting the results in the field; what seems clear, however, is that when it works, the results are often very encouraging (Mesa et al., 2017, Guarino et al., 2018). Moreover, most of the published research is focused on studying the independent effects that microbial communities and plants have on the uptake of a single pollutant, degradation and accumulation. Nonetheless, soils polluted by industrial activities rarely contain one single pollutant, and multi-pollution is common. In this study, we explored the distribution and possible functions of the microbial diversity associated with an As-tolerant *Salix atrocinerea*, growing on an abandoned brownfield and multiplied by *in vitro* techniques. In the subsequent steps, we investigated the behavior of the plants in open-air pots and in the field in the presence and absence of an endophytic (*Pantoea* sp.) or a rhizospheric (*R. erythropolis*) strain, both isolated from the same plant species growing on a TE-polluted field, and selected based on their plant-growth-promoting (PGP) traits and high As resistance.

5.2 MATERIALS AND METHODS

5.2.1 *Soil analysis*

The polluted site of Nitrastur, previously an old nitrate factory in Langreo (Asturias, Spain) has a total surface area of 20 ha. The soil for the pot experiment was collected from the two same plots where the field trial was conducted, NC5 and NC6. A scheme of the soil location and working area is shown in Mesa et al. (2017). More than half of the surface

corresponds to landfills between 4 and 5 m deep consisting of pyrite ashes and other iron and steel-type debris (Gallego et al., 2016). For measuring the total TE concentrations, four soil samples per plot of 2 m^2 were collected at a depth of 25 - 30 cm, mixed before drying in an oven at 35 °C for 72 h and sieved through a 2 mm stainless steel sieve. Then, a 100 mg soil sample was digested with 3 mL of highly-purified HNO_3 (68%) in a microwave oven and analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) as previously described in Fernández et al. (2008). To evaluate the fraction of TEs in the soil that could be potentially available for plants, the modified three-step sequential extraction procedure proposed by the European Community Bureau of Reference (BCR) was used (Rauret et al., 1999). The certified reference material BCR-701 (Sigma-Aldrich) was used for quality control purposes. The resulting fractions were analyzed by ICP-MS and the fraction of TEs available for plants was calculated as the sum of the exchangeable and reducible fractions, according to Sungur et al. (2014), whereas the non-available fraction corresponded to the sum of the oxidizable and residual fractions. Soil pH was determined by diluting 1.5 g of dry soil in 30 mL of doubled deionized water. Organic matter content was estimated by combustion and soil texture was determined by laser diffraction spectroscopy using a standardized ISO 13320 (2009) technique that provides data in the particle size range between 0.017 and 2000 mm. This procedure included disaggregation with the dispersants sodium hexametaphosphate and sodium carbonate (Sierra et al. 2008) and analysis with the Aqueous Liquid Module of the LS 13e320 MW model (Beckman Inc. Coulter).

5.2.2   *Plant/bacterial material and growth conditions for pot culture and field trial*

Plants of a clone of grey willow (*Salix atrocinerea*) were selected based on their high As tolerance and accumulation capacity. These plants originated from seeds of a tree that spontaneously grew in the TE-polluted site. Each germinated seed turned into seedlings that were cloned following the methodology previously described by Fernandez et al. (2008). The best As-accumulating *S. atrocinerea* clone (unpublished data) was selected for further experiments in pot culture and field trial. Plants of the selected clone were cultured *in vitro* in Woody Plant Medium (WPM) (pH 5.7) (Lloyd 1981), with 0.2 g L^{-1} of sequestrene 138-Fe (Ciba-Geigy AG) as iron source, 30 g L^{-1} of sucrose and 7 g L^{-1} agar. Cultures were kept in a growth chamber at 25 °C and 16 h photoperiod for 2 months.

Bacterial strains *Pantoea sp.* (AV62) and *Rhodococcus erythropolis* (AV96) were isolated from the root endosphere and the rhizosphere, respectively, of *S. atrocinerea* plants growing on the site. As already indicated, these bacteria were selected based on their IAA, ACCD, siderophore production and As resistance, and grown separately in 500 mL

Erlenmeyer flasks containing 100 mL of 869 medium with continuous shaking at 30 °C to reach 10^9 cfu mL^{-1} (24 - 48 h). Bacterial cells were recovered by centrifugation (6000 g, 10 min, 4 °C) and the bacterial pellets were re-suspended in water in numbers of 10^9 cfu mL^{-1}.

For the bacterial inoculation, before planting, plants were placed overnight in Falcon tubes containing 10 mL bacterial suspensions, ensuring proper immersion of the roots. Subsequently, 100 ml bacterial suspension were added to each plant; the same volume of water was added in the non-inoculated treatments.

For the pot experiment, *S. atrocinerea* lignified plants of 20-30 cm length were transferred to an open-air location for 6 months in 1 L pots (diameter = 14 cm, height = 14 cm) filled with a sphagnum peat:perlite mixture (3:1, v/v) (control) or TEs-polluted soil from Nitrastur and non-inoculated or inoculated with bacteria. The examined treatments were:

- *S. atrocinerea* plants in control soil, C.
- *S. atrocinerea* plants in control soil and inoculated with *Pantoea sp.*, CP.
- *S. atrocinerea* plants in control soil and inoculated with *R. erythropolis,* CR.
- *S. atrocinerea* plants in TEs-polluted soil, M.
- *S. atrocinerea* plants in TEs-polluted soil and inoculated with *Pantoea sp.*, MP.
- *S. atrocinerea* plants in TEs-polluted soil and inoculated with *R. erythropolis,* MR.

Simultaneously, a field trial was performed on a 600 m^2 experimental plot located within the industrial zone of Nitrastur for 6 months. Plants were transferred to the field and cultivated in 6 different subplots divided in subplots of 2 X 1 m with at least 1 m between plots to minimize near-neighbour effects (3 treatments x 2 replicate plots of each treatment x 12 plants in each plot). The assayed treatments were:

- *S. atrocinerea* plants in TEs-polluted soil, F.
- *S. atrocinerea* plants in TEs-polluted soil and inoculated with *Pantoea sp.*, FP.
- *S. atrocinerea* plants in TEs-polluted soil and inoculated with *R. erythropolis,* FR.

Two more bacterial re-inoculations were performed every 2 months by adding 100 ml of 10^9 cfu mL^{-1} bacterial suspension on the soil next to the base of each plant in both pot and field experiments.

5.2.3   *Plant growth and trace element concentrations*

After 6 months, plants were carefully removed from the pots, and roots exhaustively rinsed with tap water and then three times with double de-ionized water (Milli-Q 185 Plus System) at 4 ˚C. Leaves were rinsed only once in distilled water. To determine the influence of the different treatments on plant growth, fresh and dry weights and length of shoots and roots were analyzed. Three different pooled plants were separated into leaves and roots and at least three different samples per each treatment were analyzed individually. Plant material was homogenized with liquid nitrogen and stored at −80 °C until use. For plants growing on the field, a similar procedure was used but only leaves were investigated.

For determination of TE concentrations, 100 mg of oven dried plant material (at 40 ˚C for 48 h) was digested with HNO_3 in a microwave oven and analyzed by ICP-MS as described in (Fernandez et al., 2008). The following parameters were calculated: (i) Bioconcentration factor (BCF) calculated as the ratio of TE concentrations in plant root tissues and soil (BCF = C_{roots} / C_{soil}), values of BCF > 1 indicating the accumulation of a particular trace element in roots; (ii) translocation factor (TF) calculated as the ratio of TE concentrations in above-ground plant parts to those in roots (TF = $C_{above\ ground\ plant\ part}$ / C_{root}), values of TF > 1 indicating that the plant translocates TE effectively from root to shoot; (iii) mobility ratio (MR) calculated as the ratio of TEs in above-ground plant parts (shoots, branches, or leaves) to those in soil (MR = $C_{aboveground\ plant\ part}$ / C_{soil}), values of MR > 1 indicating that the plant is enriched with TEs (Lampis et al., 2015).

5.2.4   *Photosynthetic pigments, hydrogen peroxide, lipid peroxidation and free proline content in plant tissues*

Chlorophylls and carotenoids were extracted from 100 mg of frozen leaves using 10 mL of 80% acetone and analyzed as described in ***section 4.2.3.*** For the hydrogen peroxide (H_2O_2) content determination, 100 mg of frozen leaves were processed as reported in ***section 4.2.3.*** Lipid peroxidation was estimated by measuring malondialdehyde (MDA) and determining the thiobarbituric acid (TBA)-reactive compounds as described in ***section 4.2.3.*** Free proline content was determined according to ***section 4.2.3.***

5.2.5   Non-protein thiols

The extraction and analysis of non-protein thiols (NPTs) were carried out in shoots and roots of *S. atrocinerea* from 150 mg fresh samples and following the protocol described in **section 2.3.3.**

5.2.6   Total flavonoids, condensed tannins and total phenols

Samples were extracted according to Nair et al. (2012); one g fresh weight was extracted with 15 mL of methanol and the residue was extracted with 15 mL of methanol. The combined methanolic extracts were filtered and evaporated at 35 °C under reduced pressure, re-dissolved in methanol at a concentration of 10 g L^{-1}. The total flavonoid content was determined using the method of Meda et al. (2005), the condensed tannins were estimated by the vanillin method (Sun et al., 1998) and for the quantification of the total phenolics according to the Folin–Ciocalteu method as described in **section 4.2.5.**

5.2.7   Gene expression analysis in Salix atrocinerea

Isolation of RNA was carried out using the protocol described by Chang et al. (1993) with slight modifications. Frozen shoots or roots samples of 100 mg were homogenized and processed as described in **section 3.2.4**

Reverse Transcription quantitative PCR (RT-qPCR) was performed with an ABI Prism 7900HT Fast Real Time PCR system (Applied Biosystems), using Fast SYBR Green chemistry. Gene primers (**Supplementary Table 1**) were designed using Primer 3 (Untergasser et al., 2012), according to sequences of genes obtained in the Phytozome nucleotide database and described in **section 3.2.4.**

For the pot experiment, in roots *ARF2*, *EF1α* and *ACT7* were the three most stable reference genes in all the sample sets according to GrayNorm algorithm and the combination of the three was used for normalization. In shoots a combination of *ACT7*, *α-TUB2* and *EF1α* was used for normalization. For the field experiment, OTU, *EF1α and ACT7* were used for normalization.

5.2.8   Statistical analysis

To evaluate the effects of the different treatments on *S. atrocinerea* for the measured variables, a one-way or a two-way Analysis of variance (ANOVA) was performed. Data were treated and transformed when necessary as described in **section 3.2.5**. In the gene analysis

the previous was performed on both the normalized and the non-normalized data, although only the first are presented both were taking into account to establish the significance of the results. Results are expressed as the mean ± standard deviation of at least three independent replicates. All data were analyzed using R (version 3.3.1, http://www.r-project.org/) with the packages mixOmics (for PCA, version 6.0.1, http://www.mixOmics.org) and agricolae (version 1.2e4, http://tarwi.lamolina.edu.pe/~fmendiburu). Outliers were determined using the Extreme Studentized Deviate method (GraphPad Software, La Jolla, CA, USA) at significance level p ≤ 0.05.

5.3 RESULTS

5.3.1 Soil properties and TE concentration in pot and field

The physicochemical parameters of the soils used are summarized in **Table 5.1**. Nitrastur polluted soil (60 - 70% of sand particles) was classified as sandy loam with a moderately acidic pH (5.7) following the United States Department of Agriculture (USDA) specifications. The pH decreased to 4.5 once the soil was collected to perform the pot assay and homogenized in a rotatory mixer. This Nitrastur soil had no more than 3% organic matter. Lead, As, Zn, Fe and Cu concentrations were high, while concentrations of Mn, Cd and Hg were much lower (**Table 5.1**). The total As and Pb concentrations were 15 and 10-fold higher, respectively, than the Spanish regulatory limits for industrial soils (Boletin Oficial del Principado de Asturias, 2014). Most of the TEs were found in non-available fractions (especially in the residual fraction, data not shown). Both total and bioavailable TE concentrations of the control substrate (sphagnum peat:perlite mixture) were very low, in many cases even below the detection limit (**Table 5.1**).

Table 5.1. Physicochemical parameters of the soils. Data is expressed as average ± standard deviation of three different replicates. Control: (sphagnum peat:perlite mixture).

Parameters	Soil	
	Control	Nitrastur
pH	6.53 ± 0.51	4.5 ± 0.15 pot ; 5.72 ± 0.25 field
Or. matter (%)	74.23	2.95
Texture (%)		
Sand	65.27	66.32
Silt	35.13	34.76
Clay	0.60	0.22
Total metal (mg kg^{-1})		
Fe	1530 ± 113	259885.15 ± 53560.22
Mn	nd	163.8 ± 11.34
Cu	10.20 ± 0.97	1066.50 ± 130.23
Zn	14.19 ± 1.19	1368.35 ± 189.32
As	6.93 ± 0.91	2971.34 ± 310.76
Cd	nd	3.83 ± 0.35
Hg	nd	10.22 ± 0.98
Pb	17.06 ± 1.15	8401.63 ± 982.43
Available metal (mg kg^{-1})		
Fe	454.3 ± 23.8	1863.57 ± 80.23
Mn	nd	12.29 ± 0.65
Cu	4.31 ± 0.23	105.20 ±6.12
Zn	8.61 ± 0.35	81.38 ± 4.81
As	1.09 ± 0.11	162.07 ± 6.48
Cd	nd	0.22 ± 0.03
Hg	nd	1.02 ± 0.01
Pb	2.13 ± 0.20	972.26 ± 78.93

5.3.2   *Plant growth and TE accumulation in S. atrocinerea*

After 6 months of culture in pots with polluted soil, a significant decrease of shoot length was observed when compared to plants grown on control soil and this decrease was higher in non-inoculated plants (**Table 5.2**). The plants grown on polluted soil showed lower dry weight than plants grown on non-polluted soil (**Fig. 5.1A**). When plants were inoculated with bacteria in both soil types, dry weight of roots was significantly higher as compared to non-inoculated plants (**Fig. 5.1A**). Leaves from inoculated plants growing on the polluted soil (MP, MR) showed also higher dry weights than those of non-inoculated plants (M) (**Fig. 5.1A**). In the field, bacterial inoculation with *R. erythropolis* (FR) increased plant weight, especially of the leaves (**Fig. 5.1B**), whereas inoculation with *Pantoea* sp. (FP) caused a decrease in dry weight as compared to non-inoculated plants (F) (**Fig. 5.1B**).

In the pot experiment, except for Cu, leaves were the main TE storage organ in plants cultured on the control substrate (**Table 5.3**). Plants growing on a polluted soil showed higher TE concentrations in roots than in leaves for all elements except for Zn and P (**Table 5.3**) and roots from non-inoculated plants presented higher P, Fe, As and Pb concentrations than in the presence of bacteria (**Table 5.3**). In leaves, bacterial inoculation of plants in both control and polluted soils decreased the P, Fe and Zn concentrations when compared to non-inoculated plants and As as well as Pb concentrations were lower in the leaves of inoculated plants (**Table 5.3**). However, although non-inoculated plants showed the highest concentrations of TEs, there were no significant differences among treatments in the total amounts of TEs in organs (**Table 5.4**).

In the field trial, the TE concentrations were also affected by bacterial inoculation and the lowest concentrations were detected in roots and leaves of non-inoculated plants (F) (**Table 5.5**). Plants inoculated with *Pantoea* sp. (FP) contained the highest concentrations of all elements that were measured in both roots and leaves, except for Zn, which was higher in leaves of plants inoculated with *R. erythropolis* (FR) (**Table 5.5**). Owing to the low plant weight in FP (**Fig. 5.1**), F plants showed higher total element contents than FP plants, except

Table 5.2. Root and stem length (cm) of *S. atrocinerea* at 6 months of culture under different treatments in pot and field. Different letters within the same row denote significant differences on HSD test at p < 0.05.

				Size			
Treatments		C	CP	CR	M	MP	MR
Pot	Roots	36.25 ± 4.68 a	36.89 ± 4.13 a	37.44 ± 5.94 a	34.75 ± 6.75 a	33.44 ± 8.86 a	41.89 ± 7.85 a
	Stem	72.50 ± 9.40 a	61.61 ± 6.55 a	68.11 ± 12.83 a	37.11 ± 11.06 b	50.56 ± 7.84 ab	59.72 ± 11.78 ab
				Treatments	F	FP	FR
Field				Roots	21.66 ± 6.83 a	27.16 ± 10.57 b	24.53 ± 7.91 a
				Stem	22.00 ± 5.63 b	21.40 ± 5.77 b	46.43 ± 13.99 a

Table 5.3. Element accumulation (mg kg^{-1} DW) in roots and leaves of *S. atrocinerea* at 6 months of culture in pot under different treatments. Different letters within the same row and organ denote significant differences on HSD test at p < 0.05. nd: not detected.

		Treatment					
Organ	Element	Control			Polluted		
		C	CP	CR	M	MP	MR
Roots	P	748.46 ± 19.52 c	711.81 ± 5.87 d	702.45 ± 12.34 d	1477.37 ± 78.54 a	1302.53 ± 51.16 b	1247.00 ± 21.91 b
	Fe	166.03 ± 4.32 c	161.49 ± 7.33 c	172.98 ± 5.36 c	9640.60 ± 250.64 a	6437.93 ± 311.32 b	7555.59 ± 902.27 b
	Cu	14.34 ± 0.16 c	7.86 ± 0.57 d	9.75 ± 1.07 d	470.82 ± 35.35 a	416.03 ± 30.30 a	414.97 ± 45.61 a
	Zn	73.09 ± 4.00 c	62.75 ± 6.12 d	65.54 ± 6.02 cd	643.61 ± 9.64 a	621.18 ± 22.13 a	585.73 ± 52.91 a
	As	1.98 ± 0.13 c	2.28 ± 0.25 c	2.70 ± 0.11 c	199.28 ± 13.27 a	136.15 ± 15.11 b	148.17 ± 5.85 b
	Cd	0.16 ± 0.01 c	0.10 ± 0.01 c	0.15 ± 0.01 c	6.94 ± 2.34 a	5.96 ± 1.85 a	3.17 ± 1.53 b
	Hg	nd	nd	nd	4.99 ± 0.37 a	4.54 ± 0.41 a	4.25 ± 0.50 a
	Pb	0.46 ± 0.51 c	0.39 ± 0.26 c	0.64 ± 0.18 c	1064.14 ± 118.59 a	684.51 ± 45.14 b	764.75 ± 21.72 b
Leaves	P	1256.12 ± 58.91 e	1028.79 ± 61.12 d	1024.62 ± 63.09 d	1792.82 ± 106.87 a	1464.13 ± 79.34 b	1323.44 ± 81.49 c
	Fe	478.17 ± 18.54 c	344.06 ± 6.38 d	316.96 ± 68.97 d	2607.66 ± 591.91 a	1195.97 ± 22.16 b	1144.67 ± 249.07 b
	Cu	6.27 ± 0.72 c	5.54 ± 0.21 c	5.00 ± 0.33 c	28.15 ± 3.23 a	16.77 ± 0.63 b	15.09 ± 0.38 b
	Zn	207.14 ± 8.13 c	118.51 ± 20.12 d	140.46 ± 17.39 d	1728.20 ± 67.86 a	1618.04 ± 274.67 a	1271.48 ± 157.38 b
	As	0.70 ± 0.14 c	0.70 ± 0.03 c	0.68 ± 0.01 c	57.61 ± 10.46 a	25.31 ± 1.14 b	21.91 ± 5.75 b
	Cd	0.34 ± 0.02	0.68 ± 0.02	0.24 ± 0.04	4.41 ± 0.40 a	4.02 ± 0.37 a	3.41 ± 0.24 b
	Hg	nd	nd	nd	nd	nd	nd
	Pb	0.29 ± 0.50 c	0.19 ± 0.13 c	0.17 ± 0.46 c	211.55 ± 36.34 a	93.14 ± 6.49 b	74.82 ± 20.47 b
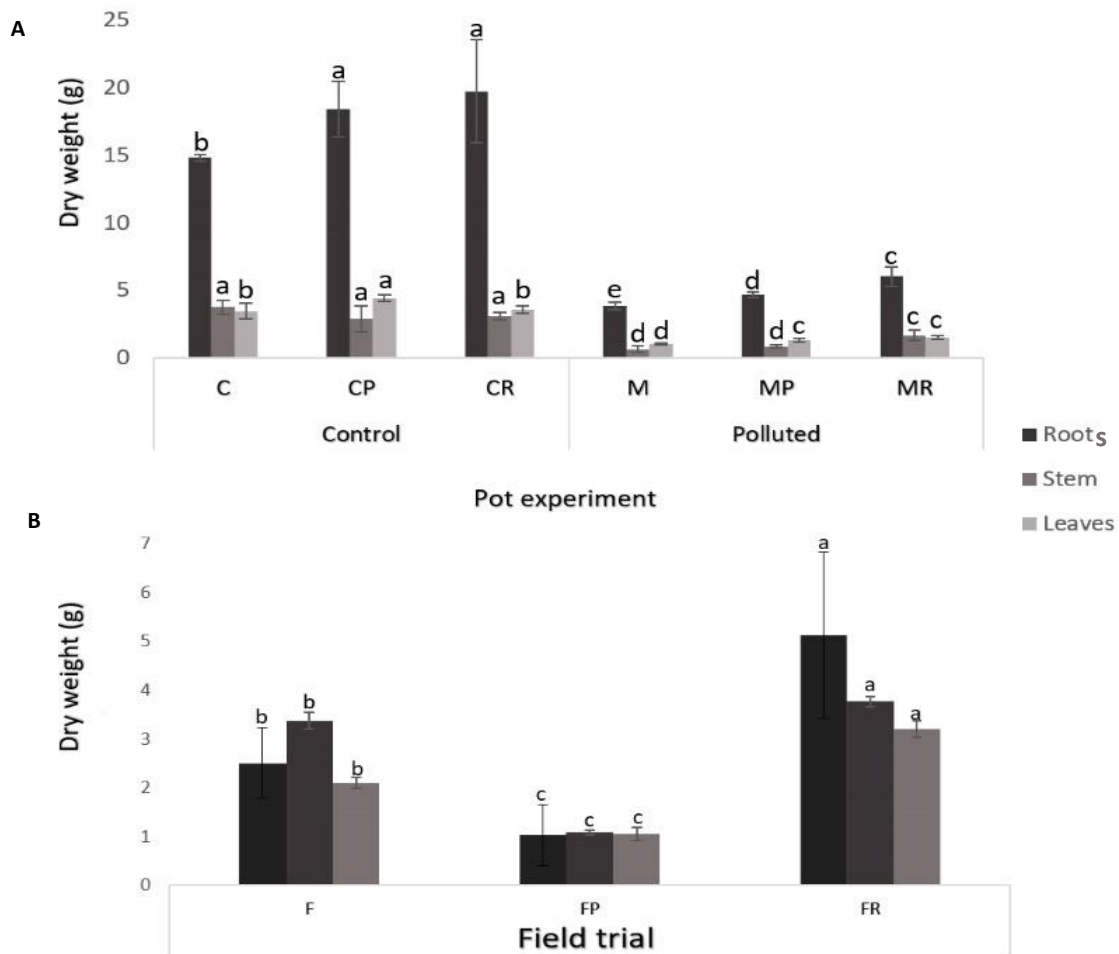
Fig. 5.1. Dry weight of roots, stems and leaves of *S. atrocinerea* at 6 months of culture in pot **(A)** and field **(B)** under different treatments. Different letters within each plant organs denote significant differences among treatments on HSD test at $p < 0.05$.

for As and Pb (**Table 5.6**), although the higher biomass achieved by FR plants (**Fig. 5.1B**), resulted in the highest TE extraction obtained in the presence of *R. erythropolis* (**Table 5.6**). Whereas Cd concentrations did not show differences between pot or field, the concentrations of Fe, Cu, Zn, As and Pb were respectively 8, 3, 4, 21 and 22-fold times higher in leaves of pot grown plants than in the field grown ones. In leaves of plants inoculated with *Pantoea* sp., Zn and As showed higher concentrations when grown in pots compared to the field (FP). Under these circumstances, P, Fe, Cu and Cd were 2, 1.6, 1.4 and 2 times higher, respectively, in leaves from the field than from pot grown plants, and no differences were observed for Pb. In the case of inoculation with *R. erythropolis*, Fe, As and Pb concentrations were higher in the leaves of plants grown in pots, whereas P and Cd were higher (1.7 and 2-fold, respectively) in the leaves of field grown plants, and no differences were observed for Cu of Zn (**Table 5.3** and **Table 5.5**).

Table 5.4. Trace element (TE) extracted (µg/per plant) in roots and leaves of *S. atrocinerea* at 6 months of culture in pot under different treatments. Different letters within the same row and organ denote significant differences on HSD test at p < 0.05. nd: not detected.

Organ	TE	Treatment		
		M	MP	MR
Roots	Fe	15075.61 ± 1000.27 a	11662.85 ± 2018.99 a	16201.70 ± 3021.87 a
	Cu	736.25 ± 49.08 a	777.59 ± 134.61 a	997.74 ± 186.09 a
	Zn	1006.45 ± 53.43 b	1314.94 ± 227.63 ab	1592.29 ± 269.99 a
	As	311.64 ± 16.54	245.60 ± 42.52	394.77 ± 73.63
	Cd	6.54 ± 0.85 a	7.37 ± 1.28 a	8.86 ± 1.65 a
	Hg	8.21 ± 0.64 a	9.44 ± 1.63 a	11.04 ± 2.06 a
	Pb	1533.01 ± 81.38 a	1397.10 ± 241.86 a	1986.06 ± 370.43 a
Leaves	Fe	1414.93 ± 245.82 a	1005.45 ± 301.69 a	1214.99 ± 386.77 a
	Cu	15.28 ± 2.65 a	14.10 ± 4.23 a	13.89 ± 4.42 a
	Zn	937.73 ± 162.92 a	1201.44 ± 360.50 a	1170.42 ± 372.58 a
	As	25.24 ± 4.39 a	21.28 ± 6.39 a	23.86 ± 7.59 a
	Cd	3.49 ± 0.61 a	3.92 ± 1.18 a	3.20 ± 1.20 a
	Hg	nd	nd	nd
	Pb	93.24 ± 16.20 a	78.30 ± 23.49 a	81.51 ± 25.95 a

Lower bioconcentration factors (BCFs) were observed for all elements in plants grown on polluted soil as compared to control soil; in field conditions, the BCFs were even lower as comparted to pot-grown plants (**Table 5.7**). In both, pots and field, Cd showed the highest BCF value (**Table 5.7**). The translocation factor (TF) of essential elements such as P, Fe and Cu was lower in plants grown on the polluted soil as compared to control soil in pots, whereas no difference was observed for Zn (**Table 5.7**). However, the TF was lower in plants grown on polluted soil than on control soil in pot (**Table 5.7**). The TF of P was higher in the field than in pots (**Table 5.7**). The mobility ratio (MR) of Cd was the highest observed for all the elements (apart from the essential nutrient P), when plants were grown on the polluted soil (**Table 5.7**).

Table 5.5. Element accumulation (mg kg^{-1} DW) in roots and leaves of *S. atrocinerea* at 6 months of culture in the field under different treatments. Different letters within the same row denote significant differences on HSD test at $p < 0.05$. nd: not detected.

Organ	Element	Treatment		
		F	FP	FR
Roots	P	1788.83 ± 137.17 b	2559.77 ± 467.72 a	1527.46 ± 108.39 b
	Fe	4928.95 ± 489.18 a	5422.04 ± 238.43 a	2577.61 ± 284.30 b
	Cu	112.99 ± 40.28 a	176.07 ± 14.42 a	110.42 ± 6.24 b
	Zn	225.82 ± 28.01 c	376.08 ± 39.56 a	305.08 ± 8.47 b
	As	28.82 ± 10.60 c	74.72 ± 10.24 a	48.17 ± 3.50 b
	Cd	3.00 ± 0.34 c	5.44 ± 0.44 a	3.56 ± 0.11 b
	Hg	0.91 ± 0.25 b	1.68 ± 0.13 a	0.93 ± 0.10 b
	Pb	178.74 ± 96.79 c	546.83 ± 115.91 a	240.10 ± 23.65 b
Leaves	P	2624.27 ± 229.55 b	3080.90 ± 58.99 a	2286.67 ± 277,97 b
	Fe	315.76 ± 32.17 c	1912.45 ± 505.29 a	608.30 ± 27.30 b
	Cu	10.43 ± 1.38 b	23.69 ± 5.25 a	13.38 ± 2.77 b
	Zn	480.94 ± 44.58 c	1153.09 ± 68.69 b	1377.19 ± 39.35 a
	As	2.71 ± 0.48 c	19.64 ± 1.64 a	6.11 ± 0.40 b
	Cd	4.75 ± 0.57 c	9.13 ± 1.52 a	8.16 ± 0.69 b
	Hg	nd	nd	nd
	Pb	9.54 ± 0.19 c	109.86 ± 17.13 a	26.03 ± 2.09 b

Table 5.6. Trace element (TE) extracted (µg/per plant) in leaves of *S. atrocinerea* at 6 months of culture in field under different treatments. Different letters within the same row denote significant differences on HSD test at $p < 0.05$. nd: not detected.

Organ	TE	Treatment		
		F	FP	FR
Roots	Fe	8573.89 ± 1593.09 b	5552.55 ± 244.17 c	13214.79 ± 1457.52 a
	Cu	265.31 ± 94.57 b	180.30 ± 14.77 b	566.09 ± 31.98 a
	Zn	530.25 ± 65.78 b	385.14 ± 40.51 c	1564.05 ± 43.43 a
	As	67.68 ± 14.89 c	86.52 ± 10.48 b	246.95 ± 17.96 a
	Cd	7.05 ± 0.79 b	5.57 ± 0.45 b	18.26 ± 0.58 a
	Hg	2.13 ± 0.59 b	1.72 ± 0.13 b	4.75 ± 0.51 a
	Pb	419.71 ± 227.28 b	559.99 ± 118.70 b	1230.92 ± 121.26 a
Leaves	Fe	592.15 ± 147.82 b	925.05 ± 209.04 b	1824.88 ± 214.64 a
	Cu	19.11 ± 4.43 b	11.46 ± 6.65 b	43.27 ± 8.95 a
	Zn	903.49 ± 225.81 b	557.75 ± 125.81 c	4828.35 ± 537.88 a
	As	4.23 ± 0.75 c	9.50 ± 2.09 b	19.77 ± 1.31 a
	Cd	7.39 ± 0.90 b	4.42 ± 1.25 c	26.40 ± 2.25 a
	Hg	nd	nd	nd
	Pb	20.19 ± 7.56 c	53.14 ± 12.49 b	84.20 ± 6.75 a

Table 5.7. Bioconcentration factor (BCF), translocation factor (TF) and mobility ratio (MR) in *S. atrocinerea* at 6 months of culture under different treatments. Different letters within the same row denote significant differences on HSD test at $p < 0.05$. nd: not detected.

	BCF								
Treatment	**Pot**						**Field**		
	Control			Polluted					
Element	C	CP	CR	M	MP	MR	F	FP	FR
P	7.38 a	7.22 a	6.83 a	2.50 e	2.38 e	2.11 f	3.03 c	4.34 b	2.59 d
Fe	0.11 a	0.10 a	0.11 a	0.04 b	0.02 c	0.03 c	0.02 c	0.02 c	0.02 c
Cu	1.40 a	0.77 c	0.96 b	0.44 d	0.39 d	0.39 d	0.11 f	0.17 e	0.10 f
Zn	5.50 a	3.72 c	4.62 b	0.47 d	0.45 d	0.42 e	0.17 f	0.27 g	0.22 h
As	0.29 c	0.33 b	0.39 a	0.07 d	0.05 d	0.05 d	0.01 e	0.03 e	0.02 e
Cd	nd	nd	nd	1.81 a	1.56 b	0.83 e	0.78 e	1.42 c	0.93 d
Hg	nd	nd	nd	0.49 a	0.44 b	0.42 b	0.09 d	0.16 c	0.09 d
Pb	0.27 b	0.23 b	0.38a	0.13 d	0.08 e	0.09 e	0.02 f	0.07 e	0.03 f
	TF								
Treatment	**Pot**						**Field**		
	Control			Polluted					
Element	C	CP	CR	M	MP	MR	F	FP	FR
P	2.17 c	2.08 d	2.18 c	1.96 d	1.73 e	1.68 e	7.13 a	7.95 a	6.30 b
Fe	3.29 a	2.70 b	2.19 c	0.32 d	0.22 e	0.18 f	0.00 g	0.01 g	0.00 g
Cu	0.82 b	1.33 a	1.05 c	0.10 d	0.09 d	0.08 d	0.03 e	0.04 e	0.03 e
Zn	3.57 a	3.46 b	3.07 c	3.55 a	3.48 b	2.94 c	0.52 f	1.06 e	1.33 d
As	0.95 a	0.84 b	0.62 c	0.34 d	0.22 e	0.17 e	0.00 f	0.01 f	0.00 f
Cd	4.14 b	10.55 a	3.16 d	1.46 f	1.51 f	2.17 e	2.26 e	4.12 b	3.76 c
Hg	nd	nd	nd	nd	nd	nd	nd	nd	nd
Pb	0.92 a	1.04 a	0.59 b	0.25 c	0.18 d	0.12 d	0.00 e	0.02 e	0.01 e
	MR								
Treatment	**Pot**						**Field**		
	Control			Polluted					
Element	C	CP	CR	M	MP	MR	F	FP	FR
P	16.00 a	15.01 b	14.92 b	4.91 e	4.12 f	3.55 f	7.90 c	7.95 c	6.30 d
Fe	0.36 a	0.27 b	0.25 b	0.01 c	0.01 c	0.01 c	0.01 c	0.01 c	0.00 c
Cu	1.15 a	1.03 a	1.00 a	0.05 b	0.03 b	0.03 b	0.04 b	0.04 b	0.03 b
Zn	19.65 a	12.86 c	14.18 b	1.67 d	1.58 e	1.24 f	1.01 g	1.06 h	1.33 f
As	0.27 a	0.27 a	0.24 a	0.02 b	0.01 b	0.01 b	0.01 b	0.01 b	0.00 b
Cd	nd	nd	nd	2.64 c	2.35 d	1.80 e	3.41 b	4.12 a	3.76 a
Hg	nd	nd	nd	nd	nd	nd	nd	nd	nd
Pb	0.24 a	0.24 a	0.22 a	0.03 b	0.01 c	0.01 c	0.01 c	0.02 c	0.01 c

5.3.3 Photosynthetic pigments

In plants growing on polluted soil in pots and in the field, chlorophyll *a* and *b* concentrations were lower, while carotenoids were higher when compared to plants grown on control soil. Chlorophyll *a* and *b* were higher in control soil inoculated with *R. erythropolis* (CR) leaves when compared to non-inoculated plants (**Table 5.8**). Anthocyanins concentrations increased in plants grown on the polluted soil in all experimental conditions as compared to plants grown on control soil with no significant differences among treatments (**Table 5.8**). In the field, no differences were observed for pigment concentrations among the treatments, whereas the plants inoculated with *Pantoea* sp. (FP) contained the lowest anthocyanins concentration (**Table 5.8**).

Table 5.8. Pigments concentration (mg g^{-1} FW) in leaves of *S. atrocinerea* at 6 months of culture under different treatments. Different letters within the same column indicate significant differences at p < 0.05.

	Treatments		Chlorophyll *a*	Chlorophyll *b*	Carotenoids	Anthocyanins
Pot	Control	C	36.05 ± 4.44 b	16.15 ± 3.86 b	7.24 ± 0.84 b	1.06 ± 0.27 e
		CP	39.54 ± 4.33 b	18.21 ± 0.83 b	7.62 ± 1.09 b	1.40 ± 0.11 d
		CR	47.95 ± 3.87 a	21.49 ± 1.09 a	7.30 ± 0.04 b	1.21 ± 0.45 de
	Polluted	M	25.43 ± 2.05 c	12.26 ± 1.23 c	10.56 ± 1.56 b	2.21 ± 0.33 c
		MP	26.05 ± 4.78 c	12.62 ± 1.92 c	10.94 ± 2.29 b	1.81 ± 0.34 c
		MR	25.25 ± 1.99 c	12.33 ± 0.92 c	13.26 ± 0.33 a	1.74 ± 0.16 c
	Field	F	18.91 ± 1.02 d	6.84 ± 1.92 d	12.69 ± 1.34 a	3.69 ± 0.43 b
		FP	22.02 ± 2.94 cd	10.72 ± 3.45 d	13.21 ± 2.45 a	2.37 ± 0.32 c
		FR	16.54 ± 3.43 d	8.63 ± 2.34 d	11.43 ± 2.91 a	5.84 ± 1.23 a

5.3.4 Parameters related to oxidative stress

In roots of plants growing in control soil in pots (CP and CR), the H_2O_2 concentration increased after inoculation; this was not the case in the leaves of the same plants (**Table 5.9**). No differences were observed in malondialdehyde (MDA) concentration among treatments in plants on control soil in pots. In general, the concentrations of H_2O_2 and MDA were higher in leaves than in roots. Plants growing on polluted soil showed higher concentrations than control plants, and values were higher in pots than in the field (Table 5.9). In the field, there were no differences in H_2O_2 concentrations among treatments, whereas MDA concentrations in the leaves of plants inoculated with *Pantoea* sp. (FP) were the highest (**Table 5.9**). No free proline could be detected in the roots in any of the pots

treatments (**Table 5.9**) and the concentrations in the leaves did not show differences among treatments (control or polluted soil; **Table 5.9**). However, in the field, *R. erythropolis* (FR) inoculated plants showed increased proline concentrations (**Table 5.9**).

Table 5.9. Hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and free proline in leaves of *S. atrocinerea* at 6 months of culture in pot and field under different treatments. Different letters within the same column indicate significant differences at $p < 0.05$. nd: not detected

Organ	Treatments			H_2O_2 (μmol g^{-1} FW)	MDA (nmol g^{-1} FW)	Free Proline (μmol g^{-1} FW)
Root	Pot	Control	C	4.41 ± 1.26 d	5.62 ± 1.15 e	nd
			CP	6.75 ± 0.74 c	6.96 ± 1.07 e	nd
			CR	5.72 ± 1.21 c	7.26 ± 0.81 de	nd
		Polluted	M	3.97 ± 0.39 d	12.60 ± 0.94 c	nd
			MP	4.06 ± 1.27 de	14.34 ± 2.41 c	nd
			MR	3.02 ± 1.08 de	12.44 ± 1.13 c	nd
	Field		F	4.96 ± 0.43 d	18.79 ± 1.23 b	nd
			FP	4.45 ± 0.81 d	18.57 ± 0.94 b	nd
			FR	3.99 ± 1.17 d	20.34 ± 0.80 b	nd
Leaves	Pot	Control	C	7.99 ± 1.53 b	17.17 ± 1.92 b	1.75 ± 0.68 b
			CP	7.05 ± 1.21 b	19.06 ± 1.44 b	1.82 ± 0.39 b
			CR	9.01 ± 0.82 b	19.33 ± 0.98 b	1.95 ± 0.39 b
		Polluted	M	12.89 ± 0.41 a	22.97 ± 1.15 a	1.68 ± 0.62 b
			MP	13.01 ± 0.86 a	21.65 ± 1.26 a	1.70 ± 0.65 b
			MR	12.28 ± 1.34 a	23.44 ± 0.87 a	1.70 ± 0.70 b
	Field		F	12.95 ± 0.50 a	7.76 ± 1.80 d	1.23 ± 0.79 b
			FP	12.58 ± 0.24 a	12.14 ± 1.15 c	2.10 ± 0.38 b
			FR	13.03 ± 0.46 a	10.87 ± 2.32 d	2.99 ± 0.11 a

5.3.5 *Non-protein thiols in plant tissues*

After 6 months of growth, changes in the concentrations of NPTs were observed in the roots and leaves of *S. atrocinerea* among the different treatments in both pot and field (**Table 5.10** and **Table 5.11**). The concentrations of NPTs were much higher in leaves than in roots (**Table 5.10**).

Roots of plants grown in control soil (C) in pots contained Cys and GSH, whereas in the contaminated soil (M) *de novo* synthesis of PC_3 was also observed (**Table 5.10**). *Pantoea* sp. inoculated plants in both CP and MP showed the highest NPTs concentration, due to increases in Cys and GSH. Leaves of plants grown in control soil contained ten different NPTs, including Cys, GSH, PC_3, PC_4, PC_6 and 5 other long chain unidentified non-protein thiolic compounds that we named as $TC_{2,3,5,7,9}$ (**Table 5.10**). Plants exposed to TEs showed *de novo* synthesis of PC_2 that together with an increase in TC_5, accounted for a higher concentration of NPTs. As seen in roots, leaves of *Pantoea* sp. inoculated plants in both CP and MP showed the highest NPTs concentration by increase of PC_6 and $TC_{5,6,7}$ (**Table 5.10**).

Table 5. 10. Non-protein thiols (NPTs) concentration (nmol GSH g^{-1} FW) in roots and leaves of *S. atrocinerea* at 6 months of culture in pot under different treatments. Different letters within the same row and organ denote significant differences on HSD test at $p < 0.05$. nd: not detected.

Organ	Thiol	Treatment					
		Control			Nitrastur		
		C	CP	CR	M	MP	MR
Roots	Cys	0.12 ± 0.01 d	0.23 ± 0.01 b	0.16 ± 0.01 c	3.12 ± 0.13 a	3.12 ± 0.08 a	3.15 ± 0.10 a
	GSH	3.84 ± 0.14 f	6.79 ± 0.41 b	4.35 ± 0.10 e	5.81 ± 0.53 c	8.20 ± 0.38 a	4.87 ± 0.10 d
	PC$_3$	nd	nd	nd	1.19 ± 0.04 a	1.00 ± 0.08 b	0.81 ± 0.02 c
	Total NTPs	4.26 ± 0.05 f	7.42 ± 0.20 d	4.82 ± 0.02 e	10.11 ± 0.26 b	12.32 ± 0.17 a	8.84 ± 0.05 c
Leaves	Cys	3.32 ± 0.23 b	1.94 ± 0.01 c	1.75 ± 0.09 d	6.16 ± 0.93 a	3.73 ± 0.21 b	3.28 ± 0.23 b
	GSH	32.42 ± 1.56 a	29.54 ± 2.12 a	28.93 ± 1.89 a	32.83 ± 1.74 a	32.57 ± 1.21 a	27.95 ± 0.38 b
	PC$_2$	nd	nd	nd	1.96 ± 0.23 ab	2.37 ± 0.18 a	1.89 ± 0.08 b
	PC$_3$	1.67 ± 0.29 a	1.08 ± 0.06 bc	1.00 ± 0.07 c	1.13 ± 0.08 bc	1.45 ± 0.05 a	1.23 ± 0.06 b
	PC$_4$	6.68 ± 0.86 ab	6.85 ± 0.60 ab	5.05 ± 0.19 c	6.94 ± 0.10 a	7.59 ± 0.54 a	5.62 ± 0.62 bc
	PC$_6$	2.99 ± 0.25 d	6.85 ± 0.60 b	5.05 ± 0.19 b	2.97 ± 0.14 d	3.63 ± 0.06 c	2.78 ± 0.15 d
	TC$_2$	1.24 ± 0.21 bc	1.07 ± 0.02 c	0.97 ± 0.04 c	0.77 ± 0.33 c	1.92 ± 0.12 a	1.44 ± 0.07 b
	TC$_3$	2.41 ± 0.27 c	2.77 ± 0.40 c	2.44 ± 0.05 c	2.06 ± 0.93 c	5.11 ± 0.21 b	15.73 ± 530 a
	TC$_5$	38.33 ± 4.13 bc	44.84 ± 7.00 ab	35.05 ± 0.37 c	35.77 ± 2.45 bc	51.22 ± 4.06 a	39.25 ± 0.74 b
	TC$_7$	62.68 ± 4.72 d	92.08 ± 3.92 b	73.13 ± 1.30 c	92.28 ± 6.11 b	128.89 ± 11.14 a	86.85 ± 7.03 b
	TC$_9$	42.30 ± 8.08 ab	49.77 ± 5.88 a	34.90 ± 0.95 b	42.13 ± 2.03 a	35.58 ± 2.10 b	29.87 ± 2.09 c
	Total NTPs	194.03 ± 2.56 e	233.37 ± 2.51 b	186.05 ± 0.62 f	225.01 ± 1.70 c	274.07 ± 3.18 a	215.88 ± 2.29 d

Field grown plants inoculated with *R. erythropolis* (FR) presented the highest NPT concentrations in roots, mainly due to the higher GSH and PC$_3$ concentrations (**Table 5.11**), whereas FP plants showed the highest NPT concentrations in leaves as a consequence of a higher TC$_{2,4,6}$ accumulations. The highest PC$_2$ concentrations were detected in leaves of FR. In general, leaves of field grown plants showed lower total NPT concentrations than in pot grown plants (mainly lower PC$_2$, PC$_4$ and PC$_6$), whereas GSH and TC$_2$ were higher in field grown plants than in pot grown plants (**Table 5.11**).

Table 5. 11. Non-protein thiols (NPTs) concentration (nmol GSH g^{-1} FW) in roots and leaves of *S. atrocinerea* at 6 months of culture in field under different treatments. Different letters within the same row and organ denote significant differences on HSD test at $p < 0.05$.

Organ	Thiol	F	FP	FR
Roots	GSH	9.19 ± 1.22 a	7.80 ± 2.53 a	11.94 ± 2.86 a
	PC$_3$	0.73 ± 0.08 b	0.63 ± 0.06 b	1.45 ± 0.13 a
	Total NTPs	9.92 ± 0.80 b	8.23 ± 1.74 b	13.39 ± 1.93 a
Leaves	Cys	3.29 ± 0.25 ab	2.77 ± 0.24 b	4.05 ± 0.67 a
	GSH	45.74 ± 3.24 a	38.08 ± 3.70 b	47.69 ± 5.35 a
	PC$_2$	1.26 ± 0.12 b	1.34 ± 0.13 b	2.96 ± 0.40 a
	PC$_4$	4.72 ± 0.72 a	4.56 ± 1.50 a	3.14 ± 1.54 a
	PC$_6$	0.81 ± 0.20 b	1.77 ± 0.86 ab	1.64 ± 0.33 a
	TC$_1$	2.85 ± 0.12 b	4.11 ± 0.88 a	2.88 ± 1.03 ab
	TC$_2$	3.59 ± 0.39 b	7.96 ± 3.63 a	4.20 ± 0.55 b
	TC$_4$	38.69 ± 3.99 b	58.18 ± 7.33 a	37.31 ± 17.11 b
	TC$_6$	15.92 ± 3.64 c	52.42 ± 7.64 a	37.40 ± 8.30 b
	TC$_8$	5.29 ± 0.44 b	7.81 ± 1.65 b	19.23 ± 6.90 a
	TC$_{10}$	3.99 ± 0.57 b	6.24 ± 1.45 a	5.70 ± 1.53 a
	TC$_{11}$	0.44 ± 0.12 b	1.21 ± 0.23 a	1.44 ± 0.40 a
	Total NTPs	126.58 ± 1.45 c	186.47 ± 2.52 a	167.64 ± 4.96 b

5.3.6 Total flavonoids, condensed tannins and phenols

In leaves of plants grown in polluted soil in pots, the total flavonoid content was lower than the control, especially when inoculated with *R. erythropolis* (**Fig. 5.2A**). The leaves of inoculated plants grown on control and polluted soil, showed higher contents of condensed tannins than non-inoculated plants (**Fig. 5.2A**). Total phenols were not different between soil types and among treatments (**Fig. 5.2A**).

Field grown plants inoculated with *Pantoea* sp. showed higher flavonoid concentrations compared to the non-inoculated ones (**Fig. 5.2B**). Field grown inoculated plants, especially with *R. erythropolis*, showed higher concentrations of both, condensed tannins and total phenols (**Fig. 5.2B**).
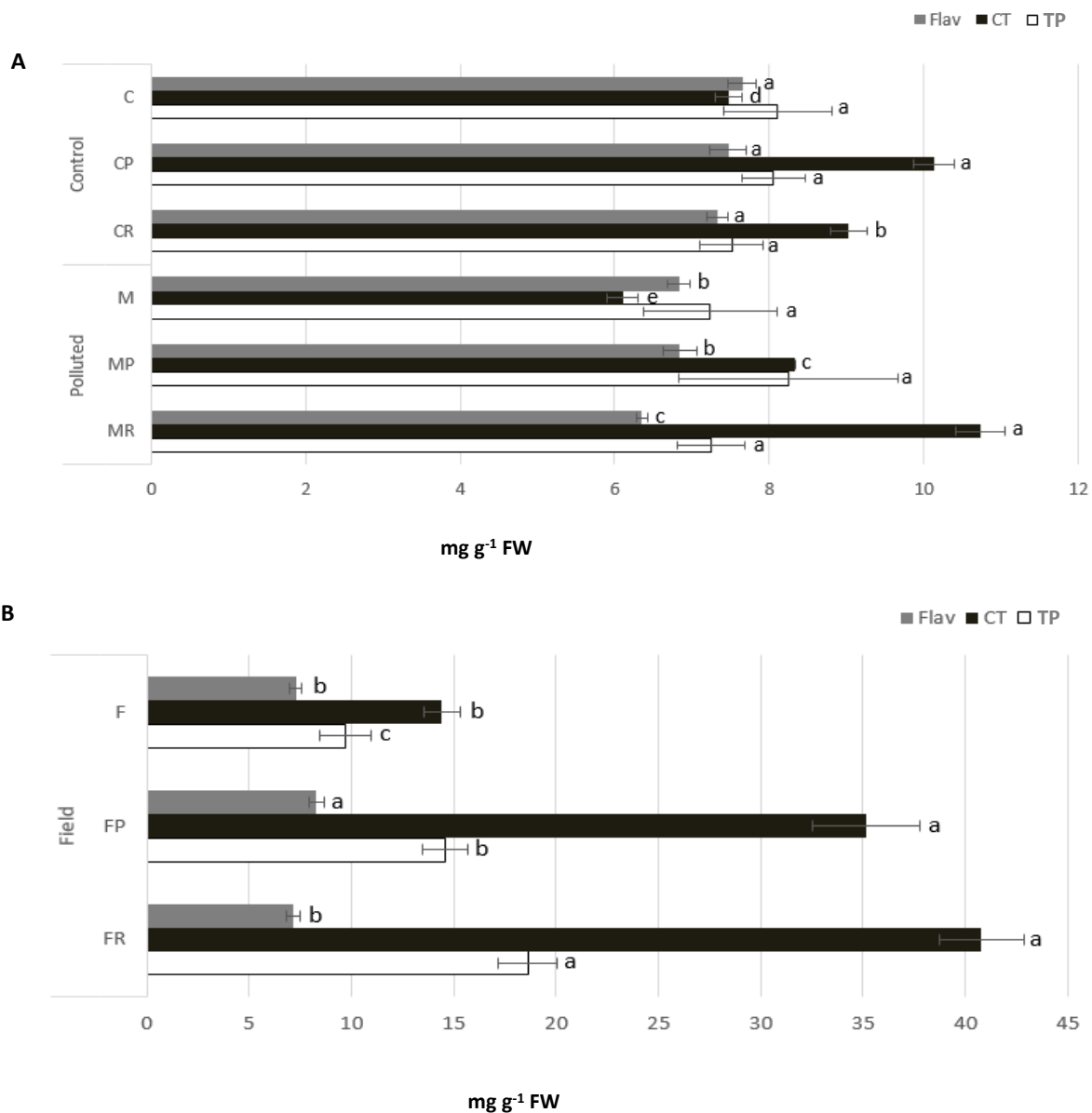
Fig. 2. Total flavonoids (Flav), condensed tannins (CT) and total phenols (TP) in leaves of *S. atrocinerea* at 6 months of culture under different treatments in pot (**A**) and field (**B**). Different letters within each compound indicate significant differences among treatments on HSD test at p < 0.05.

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5.3.7   *Gene expression*

5.3.7.1   *Differential gene expression in roots*

In the pot grown plants, clear differences were observed in gene expression patterns between *S. atrocinerea* plants growing on the control (C) and polluted soil (M). In roots of M plants the transcripts for aquaporins transporters (*SIP.1* and *SILICON*) and metallothioneins (*MT1A*) were 1.7-fold higher than in control plants; a 2-fold down-regulation was observed for transcripts levels of arsenate reductase (*CD25-1*) (**Fig. 5.3A**). Transcripts coding for the ethylene receptor protein (*ER*) and aminocyclopropane-1-carboxylate synthase (*ACCS*) were up-regulated 2 (**Fig. 5.3A)** and 16-fold (**Table 5.12**), respectively, whereas transcripts coding for cellulose synthase (*CSA*) were 2-fold down-regulated (**Fig. 5.3A)**. The effects of bacterial inoculation on metal tolerance transcripts were reflected in the control soil, by 1.5 and 1.7-fold down-regulations of transcripts associated with a high-affinity phosphate transporter (*PHO1*) in plants inoculated with *Pantoea* sp (CP) and *R. erythropolis* (CR), respectively, a 2-fold down-regulation of transcripts encoding for another high-affinity phosphate transporter protein (*HAPO4*) in CP and CR, a 3-fold down-regulation of an arsenite-inducible chaperone (*AIP-1*) in CR, 1.7 and 1.5-fold up-regulations of transcripts for a phytochelatin synthetase-like (*PCS-L*) in CP and CR, respectively (**Fig. 5.3B**), 2.6 and 2.4-fold up-regulations of transcripts encoding for a vacuolar transporter (*ABCG2*) in CP and CR, respectively, and 5.2 and 3.2-fold up-regulations for a white-brown-complex ABC transporter (*WBABCT*) in CP and CR, respectively (**Table 5.12**). In roots of plants grown on the polluted soil, transcripts for glutathione reductase (*GR*) were 1.5 fold up-regulated in MP and MR as compared to M plants. A 1.8-fold up-regulation of *ABCG2* in MP and MR and 1.6 and 1.7-fold up-regulations of *WBABCT* in CP and CR, respectively were found (**Fig. 5.3C**). Finally, the metal stress-related gene *AIP-1* was down-regulated in MP and MR (3.8 and 2.8-fold, respectively) as well as the *ER* gene (2 and 2.2-fold, respectively) (**Table 5.12**).

5.3.7.2   *Differential gene expression in leaves*

The presence of pollutants in the soil and their translocation and accumulation in aboveground plant tissues triggered also differences in transcript levels of several genes in leaves the of *S. atrocinerea*, including As-related transporters. In leaves of plants grown on polluted soil (M plants), transcripts associated to the phosphate transporters *PHO1* and *HAPO4* were 1.6 and 2-fold up-regulated, respectively (**Fig. 5.4A**). Arsenite transport-related proteins, as the transcripts encoding the aquaporins transporters NIP1.1, SIP.1 and
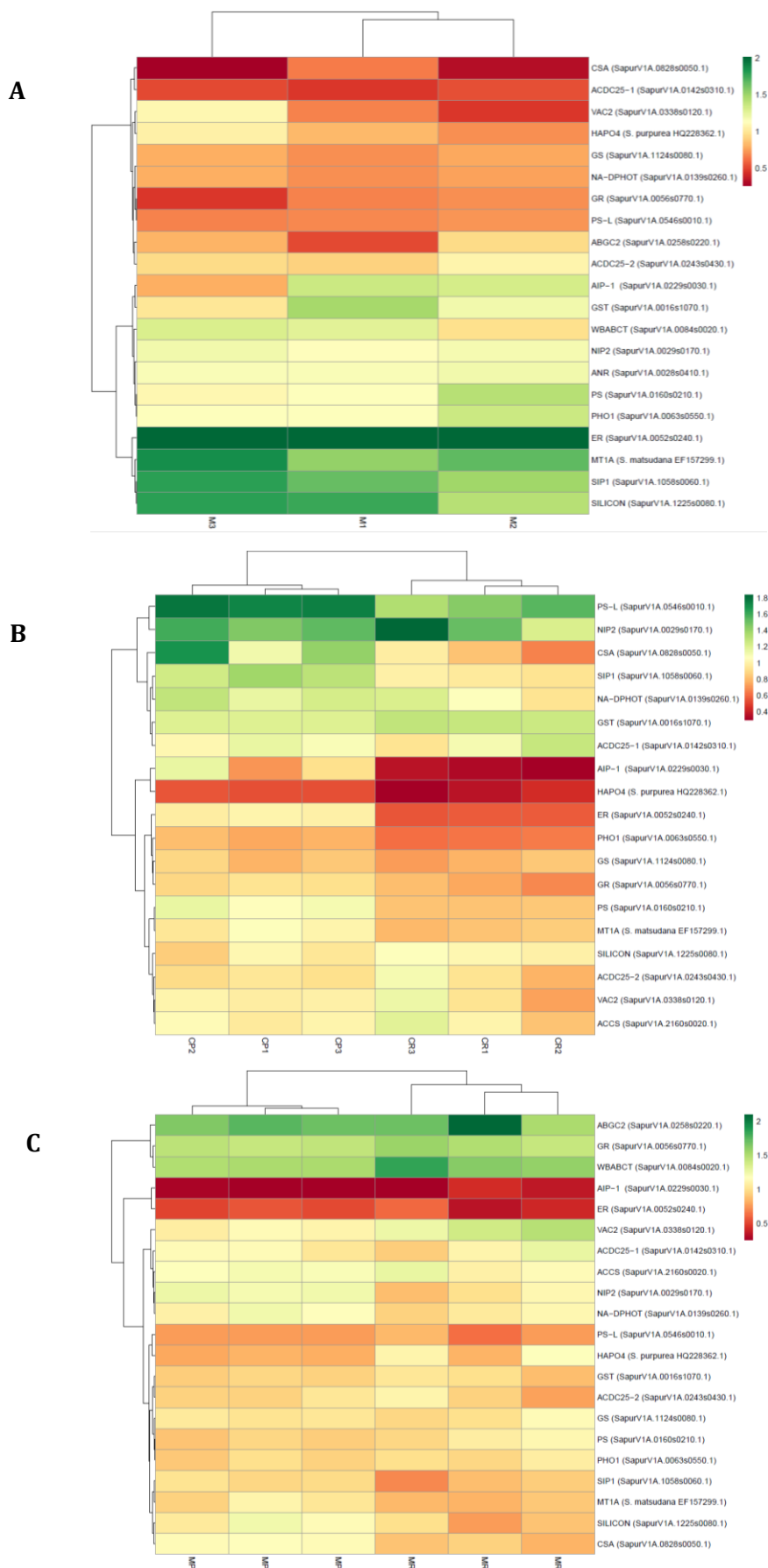
Fig. 5.3. Heat map representations of the gene expression data of samples of roots of *S. atrocinerea* at 6 months of culture in plants grown in a polluted soil (M) relative to non-exposed plants (**A**), inoculated plants with *Pantoea* sp. (CP) and *R. erythropolis* (CR) grown in a control soil relative to non-inoculated (**B**) and inoculated plants with *Pantoea* sp. (MP) and *R. erythropolis* (MR) grown in a polluted soil relative to non-inoculated (**C**). Hierarchical clustering is based on the most differentially expressed genes with a fold regulation equal or lower than 2. Gene expression level values are the normalized expression relative to the non-exposed plants at each time point of three biological replicates, each containing at least one individual plant. Green-shaded rectangles indicate increased, while red-shaded rectangles indicate decreased gene expression. For gene abbreviations see *Supplementary Table 1.*

Fig 5.4. Heat map representations of the gene expression data of samples of leaves of *S. atrocinerea* at 6 months of culture in plants grown in a polluted soil (M) relative to non-exposed plants (**A**), inoculated plants with *Pantoea* sp. (CP) and *R. erythropolis* (CR) grown in a control soil relative to non-inoculated (**B**) and inoculated plants with *Pantoea* sp. (MP) and *R. erythropolis* (MR) grown in a polluted soil relative to non-inoculated (**C**). Hierarchical clustering is based on the most differentially expressed genes with a fold regulation equal or lower than 2. Gene expression level values are the normalized expression relative to the non-exposed plants at each time point of three biological replicates, each containing at least one individual plant. Green-shaded rectangles indicate increased, while red-shaded rectangles indicate decreased gene expression. For gene abbreviations see ***Supplementary Table 1.***

SILICON, were 1.6, 2 (**Fig. 5.4A**) and 3.4-fold up-regulated (**Table 5.12**), respectively. Transcripts involved in arsenic reduction in leaves, *CDC25*-like tyrosine phosphatase *CDC25-1* and *CDC25-2* showed a 2-fold down-regulation and a 1.5-fold up-regulation, respectively (**Fig. 5.4A)**. The genes involved in thiol metabolism, such as the transcripts for glutathione S-transferase (GST) and phytochelatin synthetase (PCS) showed a 1.5-fold up-regulation, whereas transcripts for PS-L were 2-fold down-regulated (**Fig. 5.4A**). A 3-fold up-regulation was observed for *MT1A* (**Table 5.12**). Vacuolar transport dynamics of PC-metal complexes in leaves of plants grown in polluted soil was represented by a 52-fold up-regulation of *WBABCT* transcripts (**Table 5.12**). Metal stress-related genes, *ACCS* and *ER* showed a 3.5 and 5.5-fold up-regulation, respectively (**Table 5.12**). In contrast to roots, no changes where observed in *CSA* (**Fig. 5.4A**). Transcripts encoding enzymes regulating the phenylpropanoid and flavonoid pathways were differentially expressed in leaves of plants exposed to metal(loid)s. The three transcripts encoding a chalcone synthase (CHS) starting the enzyme pathway, *CHS1*, *CHS3* and *CHS2*, were 2 (**Fig. 5.4A**) and 3-fold up-regulated, and 2.3-fold down-regulated, respectively (**Table 5.12**). Regarding transcripts coding for the rest of the pathway, like anthocyanidin synthase (ANS) and flavonol synthase (FLS), 5.7 (**Table 5.12**) and 1.5-fold (**Fig. 5.4A**) up-regulations were observed, respectively. Transcripts coding for flavonoid 3'-hydroxylase (FLH), flavanone 3-hydroxylase (F3H) and dihydroflavonol 4-reductase (2HFLR) were 2.8 (**Table 5.12**), 2 and 1.5-fold up-regulated, respectively (**Fig. 5.4A**).

The effects of bacterial inoculation on gene expression in leaves differed in function of the soil the plants were growing on. In leaves of control soil grown plants, there were changes in transcripts encoding for As-related transporters. *PHO1* was respectively 1.5 and 1.9-fold down-regulated in the presence of *Pantoea* sp. (CP) and *R. erythropolis* (CR) (**Fig. 5.4B**). Transcripts for HAPO4 and for a sodium-dependent phosphate transporter (NA-DPHOT) were 1.5-fold down-regulated in leaves of CR plants (**Fig. 5.4B**), and transcripts for SILICON were 2-fold up-regulated in CP (**Fig. 5.4B**). The thiol metabolism related gene *PS* was 1.5 and 2-fold down-regulated in CP and CR, respectively, whereas *PS-L* and *GR* were down-regulated in CR, 2.5 (**Table 5.12**) and 1.5-fold (**Fig. 5.4B)**, respectively. In leaves of CR plants, 2.6, 2 and 2-fold down-regulations were observed for transcripts coding for the vacuolar transporters WBABCT (**Table 5.12)**, ABCG2 and VAC2, respectively (**Fig. 5.4B**). Up-regulations of genes related with the phenylpropanoid pathway were observed after bacterial inoculation: *CHS1* (5.4 and 3.6-fold), *CHS3* (7.1 and 4.9-fold), *F3H* (2.2 and 1.7-fold) and *ANS* (8.7 and 9.4-fold) in leaves of CP and CR plants, respectively. In CP, *FLH* and anthocyanidin reductase (*ANR*) were up-regulated 2.6 and 1.9-fold, respectively (**Table 5.12**). In plants grown on the polluted soil, there was a general down-regulation for all

genes measured in the plants inoculated with *Pantoea* sp. (MP) and more markedly in those inoculated with *R. erythropolis* (MR) (**Fig. 5.4C** and **Table 5.12**), with the exception of a 2-fold up-regulation of *CHS2* in both MP and MR (**Fig. 5.4C**) and unchanged transcript levels for *CSA*, *ER* or *ACCS* (**Fig. 5.4C**).

Fig 5.5. Heat map representations of the gene expression data relative to non-inoculated plants (F) of samples of leaves of *S. atrocinerea* inoculated with *Pantoea* sp. (CP) or *R. erythropolis* (CR) at 6 months of culture in the field. Hierarchical clustering is based on the most differentially expressed genes with a fold regulation equal or lower than 2. Gene expression level values are the normalized expression relative to the non-exposed plants at each time point of at least three biological replicates, each containing at least one individual plant. Green-shaded rectangles indicate increased, while red-shaded rectangles indicate decreased gene expression. For gene abbreviations see ***Supplementary Table 1.***

In field grown plants (F), changes in differential regulation of transcripts were observed in the leaves of inoculated plants with respect to non-inoculated plants. For example, in plants inoculated with *R. erythropolis* (FR) a 2-fold up-regulation in transcripts for *HAPO4* was observed. Arsenite transport-related *SILICON* was 2-fold up-regulated in leaves of plants inoculated with both, *R. erythropolis* (FR) and *Pantoea* sp. (**Fig. 5.5**). (FP), and *ACCS* transcripts were 2.7 and 2-fold up-regulated in FP and FR, respectively. In contrast, 2 and 1.6-fold down-regulations were seen for *CSA* transcripts, in leaves of FP and FR plants, respectively (**Fig. 5.5**). Concerning thiol metabolism-related genes, there was a 1.6-fold increase of *GS* transcripts in FP leaves and 1.7-fold up-regulation of *GR* transcripts in FR leaves. Metallothioneins *MT1A* transcripts were 1.7-fold up-regulated in FR (**Fig. 5.5**). Vacuolar transport transcripts *WBABCT* showed a 1.7-fold up-regulation of expression in FP (**Fig. 5.5**), and *ABCG2* transcripts showed a 2 and 2.5 fold up-regulation in FP and FR

leaves, respectively (**Table 5.12**). Transcripts encoding enzymes regulating the phenylpropanoid pathway were differentially expressed in response to bacterial inoculation; *CHS* transcripts were up-regulated in the presence of bacteria, with the exception of *CHS1* in *Pantoea* sp. (FP) (**Table 5.12**). For the rest of the pathway, *F3H* was 1.6 and 2.5-fold up-regulated in FP and FR leaves, respectively (**Table 5.12**), whereas *FLH* was 1.7-fold up-regulated only in FR leaves (**Fig. 5.5)** and *ANS* transcripts were 3.8 and 10.4-fold up-regulated in FP and FR leaves, respectively (**Table 5.12**).

Table 5.12. Relative gene expression levels in roots and leaves of *S. atrocinerea* at 6 months of culture in pot and field under different treatments of those genes with a regulation higher than 2-fold. Values are mean normalized expression relative to the plants grown in control soil or non-inoculated at each time point (set at 1.00) ± S.D. of at least three biological replicates, each containing at least one individual plant. Statistically significant at p < 0.05 As-induced changes in expression relative to the non-exposed plants at each time point are indicated by color (■ = upregulation; ■ = downregulation). Different letters or asterisks within each row and plant tissue indicate significant differences among treatments for each experiment on HSD test at p < 0.05.

Organ	Function	Gene	Pot — relative to non-inoculated plants in control soil			Pot — relative to non-inoculated plants in polluted soil		Field — Relative to non-inoculated plants	
			M	CP	CR	MP	MR	FP	FR
Roots	Vacuolar transporter	WBABCT		5.17 ± 1.12 a	3.24 ± 0.73 a				
		ABCG2		2.59 ± 0.50 a	2.55 ± 0.60 a				
	Stress	AIP-1				0.27 ± 0.08	0.36 ± 0.08		
		ACCS	15.90 ± 0.84						
		ER				0.53 ± 0.15	0.45 ± 0.15		
Leaves	Transporters	PHO1				0.44 ± 0.04	0.34 ± 0.00		
		SILICON	3.68 ± 0.80						
	Sulphur metabolism	GS				0.61 ± 0.04	0.39 ± 0.04		
		GST				0.63 ± 0.05	0.42 ± 0.07		
		PCS				0.61 ± 0.02	0.41 ± 0.04		
		PCS-L		0.92 ± 0.09 a	0.40 ± 0.03 b				
		MT1A	3.31 ± 0.91						
	Vacuolar transporter	ABCG2						2.17 ± 0.05	2.50 ± 0.25
		WBABCT	51.47 ± 0.59	0.80 ± 0.06	0.38 ± 0.02				
		VAC2				0.53 ± 0.04	0.39 ± 0.05		
	Stress	ACCS	4.07 ± 1.24					2.67 ± 0.50	2.02 ± 0.41
		ER	4.79 ± 1.37						
	Flavonoid pathway	CHS1		5.36 ± 1.08	3.57 ± 0.40	0.72 ± 0.20	0.27 ± 0.05	0.88 ± 0.13	3.21 ± 1.04
		CHS2						1.53 ± 0.13	3.20 ± 0.93
		CHS3	4.53 ± 2.65	7.12 ± 1.37	4.85 ± 0.79	0.50 ± 0.09	0.36 ± 0.16	1.65 ± 0.35	2.97 ± 0.94
		FLH	2.82 ± 0.42	2.66 ± 0.20	1.31 ± 0.12				
		F3H		2.24 ± 0.43	1.74 ± 0.12			1.62 ± 0.13	2.52 ± 0.85
		ANS	7.55 ± 3.93	8.67 ± 2.57	9.39 ± 0.76	1.08 ± 0.05	0.27 ± 0.04	3.77 ± 1.18	10.37 ± 0.57

5.4   DISCUSSION

Phytoremediation of TE-polluted soils is an eco-friendly and therefore a highly recommended remediation approach (Ma et al., 2016; Jacob et al., 2018;) and *Salix* is a genus that can be used in phytoremediation processes. Native shrubby willow is a tolerant species that grows spontaneously in the polluted soils. It has been suggested that the reported accumulation of TEs, especially for As, coupled with high biomass yields, makes willow species attractive for phytoremediation (Landberg and Greger, 2004; Witters et al., 2009; Ruttens et al., 2011; Van Slycken et al., 2013; Gonzalez et al., 2015; Yanitch et al. 2017; Thijs et al., 2018). The genetic diversity associated with rapid growth and peculiar physiological and biochemical characteristics are important requirements for the selection of genotypes useful for phytoremediation (Pulford and Watson, 2003). Moreover, Vysloužilová et al. (2003), Tlustoš et al. (2007) and Jensen et al. (2009) showed that when *Salix* grows in moderately polluted soils - according to current legislation - a high removal of TEs takes place, while phytoextraction potential is low in extremely polluted soils due to a decline in the biomass production, a fact that coincides with the results obtained in our work (**Fig. 5.1**). In relation to this, the down-regulation of cellulose synthase gene *(CSA)* in roots (**Fig. 5.3A**) is in agreement with the results observed in rice by Huang et al. (2012b), suggesting that TE accumulation and the stress induced by it, could limit cell expansion and thus, plant growth (Le Gall et al., 2015).

In the polluted soil studied, total concentrations of several TEs were high as compared to legislated values, being the concentrations of Zn, Cu, Pb, and As about 6, 8, 44, and 93 times higher, respectively, than the maximum threshold values in agricultural soils (Kabata-Pendias, 2010). Despite this, sequential fractionation analysis (BCR method) suggested that more than 90% of the Fe, Cu, Zn, As, Cd, and Hg was present in the non-available fractions, although the available fractions of these elements were large enough to necessitate soil remediation (Kabata-Pendias, 2010). Only the TE amount present in these fractions can be easily taken up by roots or can become available under influence of various chemical and/or biological processes (Li et al., 2010). Lead was the element with the highest bioavailable concentration (20%) and taking into account its high toxicity, this element, together with As, represents the highest environmental risk in Nitrastur soil, with both Pb and As being accumulated in *Salix* at toxic concentrations for plant tissues (White and Brown, 2010).

The study of the importance of physio-chemical properties in driving soil bacterial communities has received much attention in the last decades (Wu et al., 2018). In our case, the sandy texture of the polluted soil stimulates bacterial growth and development, since the low fertility increases the dependence of plants on microbial

associations to obtain some essential elements (Jacoby et al., 2017). The growth of soil microbes is usually carbon-limited, so the high amounts of sugars, amino acids, and organic acids that plants excrete to the rhizosphere represent a valuable nutrition source for microorganisms (Bais et al., 2006). Another crucial factor affecting microbial communities in soil is pH, which can strongly influence abiotic factors, such as the solubility of TEs, availability of carbon and nutrients and biotic factors, such as the biomass composition of fungi and bacteria (Fierer and Jackson, 2006; Rousk et al., 2009). As it has been observed by Baath (1998), bacterial growth increases with raising pH, and, in our case, the fact that the pH in the mixed polluted soil used in the pots was lower than the one observed in the field could explain, among other factors, some of the differences found between effects of inoculations in pot and field assays. Another possible cause to explain the different outcomes from pot and field experiments is that the respiration of microorganisms is strongly related to soil temperature (Bradford et al., 2008), as well as the nutrient requirements for plant growth (Jakobsen et al., 2001; Watt et al., 2006). Soil texture may equally affect the rhizosphere microflora by limiting the availability of root exudates and oxygen. In our case, the sandy texture of the polluted soil, is characterized by its porosity and good aeration, which is favoring nutrient exchange and a root exudation, as well as oxygen exchange (Garbeva et al., 2004).

One of the major issues for plant nutrition is the availability of nutrients in the soil (van der Heijden et al., 2008; Ayangbenro and Babalola, 2017). This applies also to pollutants, and limited TE availability in soils is the prime factor to restrict the applicability of phytoremediation. It has also been demonstrated that in some cases inoculation of PGP bacteria helps the plant to overcome TE-induced toxicity by improving the nutritional status and reducing uptake and translocation of toxic TEs in plants (Ahmad et al., 2015). This can be achieved by bacterial transformation of the TEs to less toxic forms and/or by enhancing plant biomass due to metabolite production (Pandey et al., 2018). Bioaugmentation by inoculation of *Pantoea* sp. and *R. erythropolis* in the polluted soil decreased concentrations of TEs in roots and leaves of *S. atrocinerea* plants growing in pots. However, an opposite trend was observed in the field trial. In the pots, bacterial inoculation increased the biomass of *S. atrocinerea*, probably by lowering plant TE uptake, although the total amount of TEs extracted by the inoculated plants was not significantly different from the non-inoculated one (**Table 5.4**). On the long term, inoculated plants could be able to extract a higher quantity of TEs than non-inoculated plants. The increased plant biomass and stress tolerance of willow can also be attributed to PGP compounds produced by the bacterial strains used. However, inoculation with *Pantoea sp.* in the field led to higher TE concentrations in plant tissues and higher phytotoxicity, which caused a lower biomass

(**Fig. 5.1**). As a consequence, less P, Cu, Zn and Cd was extracted compared to non-inoculated plants (**Table 5.6**). On the contrary, although inoculation with *R. erythropolis* in the field led to a lower TE concentration in willow than with *Pantoea* sp. (**Table 5.5**), higher TE phytoextraction was obtained due to a higher plants biomass production (**Fig. 5.1**). Rosselli et al. (2003) found a better correlation between the results of pot and field experiments when studying the phytoextraction potential of fast growing trees. Nevertheless, concentration factors for Cu, Zn, and Cd were higher in pot experiments. They explained these results by the restricted volume of soil prospected by the roots and thus better ion uptake. In contrast to this study, Janssen et al. (2015) revealed a two to tenfold higher TE uptake (Cd, Zn, Pb, Cu) in field trials in comparison to TE uptake by willow plants in growth-chamber experiments. Altogether, these results highlight the complexity of soil-microbe-plant interactions and the need for more studies.

A large number of studies highlighted that among the fast-growing trees, *Salix* species are the best candidates for the removal of Cd (Hermle et al., 2006; Evangelou et al. 2013). Although the availability of Cd was very low in the Nitrastur soil as compared to legislation values for contaminated soils, our results showed that *S. atrocinerea* could accumulate higher concentrations of Cd in roots and leaves than that present in the soil (**Table 5.3** and **Table 5.5**). Moreover, the Zn concentrations were also high, especially in the leaves. It has been proposed that the high Zn translocation factor to aerial parts might be a mechanism to eliminate the metal from the plant, via leaf fall in autumn (Dahmani-Muller et al., 2000). Like in other studies, the highest concentrations of As, Cu and Pb were found in the roots (Lunackova et al., 2003; Dickinson and Pulford, 2005; Tlustoš et al., 2007, Pajević et al., 2016). Concerning As, studies with different plant species (Zhao et al., 2009; Mesa et al., 2017, Yanitch et al., 2017) reported that translocation from roots to shoots is generally low, which agrees with the high As concentrations found in roots and low translocation observed in *S. atrocinerea*. In other studies, willows showed low As uptake and accumulation in aboveground tissues (Tlustoš et al., 2007; Laidlaw et al., 2012¸Pajević et al., 2016). In our case, the accumulation of As in the aboveground parts of potted plants was 36 % taking into account the available As concentration of the soil and only 12% in the field in the presence of *Pantoea* sp, despite the fact that this treatment showed the highest As concentration in leaves. This indicates that in field experiments, due to a greater number of unpredictable and/or difficult to control factors, the results are different from those obtained in hydroponic and pot experiments. In any case these environmental conditions likely provide more realistic data of the phytoextraction potential in the long term (Li et al., 2012).

The decreases in photosynthetic pigments in the leaves of *S. atrocinerea* plants cultivated in polluted soil agree with the reports of photosynthetic pigments being one of

the sensitive points for TE toxicity (Azizur Rahman et al., 2007). Even low amounts of TEs reaching the chloroplast may affect pigment biosynthesis through inhibition of tetrapyrrole biosynthesis (Mishra et al., 2014; 2016). Long-term exposure causes reduced leaf biomass and decreases of photosynthetic pigments (**Table 5.8**), accompanied by a decrease in activities involved in CO_2 assimilation and growth (Shi et al., 2010). The fact that inoculation with *R. erythropolis* increased the concentration of photosynthetic pigments in leaves of plants grown on control soil by increasing contents of chlorophyll *a* and *b*, and in polluted soil by increasing charotenoids concentration (**Table 5.8**), coincides with other bacterial inoculation studies (Campos et al., 2015; Castanheira et al., 2017), in which improvements of the photosynthetic apparatus and anti-oxidative capacity have been suggested.

Anthocyanins can be produced in response to TE stress (Hale et al., 2001) and are believed to increase the antioxidant response of plants in order to support the regular physiological defense against abiotic and also biotic stresses (Neill et al., 2002). In our study, increased anthocyanins concentrations were found after TE exposure of plants in pots (**Table 5.8**). This was supported by the increased gene expression of *ANS* (**Table 5.12**). In contrast, we observed that higher TE accumulation in the field (FP) was accompanied by a decrease in anthocyanins. This could be due to an increased effect of TE toxicity (Krupa et al., 1996); however, in general terms, anthocyanins concentrations were higher in field grown plants than in pot grown plants (**Table 5.8**). Therefore, possible influences of additional abiotic or biotic factors other than metal accumulation should be also considered (Appelhagen et al., 2018). For example, bacterial inoculation in control soil grown plants could eliminate the effect of toxic metals leading to higher anthocyanin concentrations. It is known that beneficial soil microorganisms, like bacteria and arbuscular mycorrhizal (AM) fungi can influence the plant secondary metabolic pathways (Zeng et al., 2013) and that after bacterial infection, anthocyanins play a protective role against damage by ROS (Kangatharalingam et al., 2002). Therefore, in plants grown under control conditions, an up-regulation of *ANS* transcripts can be related to an infection response, while a down-regulation in inoculated plants compared to non-inoculated plants, can correspond to lower TE concentrations in plant tissues and a lower cellular stress status.

The changes observed in our work in H_2O_2 content of plants under TE exposure, agree with earlier evidence for a correlation between H_2O_2 content and plant metal tolerance (Cuypers et al., 2016). The higher TE concentrations observed in *Pantoea* sp. inoculated plants in the field (FP) coincides with a higher MDA content (**Table 5.9**), which is often used as an indicator of oxidative damage during peroxidation of membrane lipids by decomposition of polyunsaturated fatty acid (Mittler, 2002). Since the concentrations of the most abundant toxic elements in the soil, As and Pb, were higher in leaves of plants growing

in pots than in the field, it is not surprising that plants growing in pots showed higher MDA concentrations than the plants growing in the field. Proline is a well-known osmoprotectant that is accumulated in plants under metal stress (Mohd et al. 2017; Abbas et al. 2018). Decreases of proline in plants were reported after inoculation with PGP bacteria and were correlated with stress mitigation by these bacteria (Barnawal et al., 2012). Since plants inoculated with bacteria in the field accumulated more Fe, Cu, Zn, As, Cd and Pb than non-inoculated plants, and proline levels were higher in the inoculated plants, we might conclude that proline may be induced above a certain threshold concentration of TEs in *S. atrocinerea*. Also in relation to stress adaptation, the up-regulation of *ER* and *ACCS* transcripts in both roots and leaves (**Table 5.12**) is in accordance with several studies that have reported the involvement of ethylene in response to TEs (Maksymiec, 2007; DalCorso et al., 2010; Yanitch et al., 2017). However, the way in which ethylene is involved in mechanisms of TE responses remains unclear (Schellingen et al., 2015; Keunen et al., 2016; Bücker-Neto et al., 2017).

The transcriptional changes induced by exposure to non-essential toxic TEs help to understand the plant tolerance mechanisms to TE-induced toxicity and the role that plant-associated bacteria may play (Herbette et al., 2006; Mendoza-Cózatl et al., 2011). Arsenic and Pb are the most important soil pollutants in Nitrastur in terms of total concentration and, therefore, it was expected that plant would accumulate both at high concentrations. However, this was not the case and other pollutants were accumulated at higher concentrations. Nonetheless, except for the peculiarities related to As speciation in tissues and the precise toxicity mechanism of every metal(loid), TE accumulation at toxic concentrations in plant tissues causes a similar response based on synthesis of specific low-molecular-weight chelators, TE complexation, sequestration into vacuoles and maintaining protein stability, *etc.* Therefore, the study of a similar set of genes as those previously used for As in **Chapter 3** and **Chapter 4** is suitable.

Roots are the first point of contact of the plant with the toxic elements present in the soil. Therefore, increased *SIP.1* and *SILICON* expression in roots of *S. atrocinerea* exposed to TEs, were expected since aquaporins intervene in H_2O_2 transport and TE homeostasis (Noronha et al., 2016). Whereas no changes were observed for *GS* transcripts, roots of plants grown in the polluted soil contained higher GSH concentrations than those growing under control conditions (**Table 5.10**), which highlights the importance of this thiol in TE detoxification. GSH concentrations were higher in field than in pot grown plants, which suggests other roles of GSH in TE tolerance than just metal chelation. Besides being a precursor for synthesis of PCs, GSH also plays a role in metal chelation, the control of gene transcription, and the maintenance of the cellular redox state (Jozefczak et al., 2012;

Zagorchev et al., 2013). Most of the other NPTs detected in *S. atrocinerea* plants grown in polluted soils were also present in the control plants, which supports the hypothesis that, in plant cells, NPTs also play a role in the maintenance of metal ion homeostasis (Vurro et al., 2011; Akhter et al., 2012; Fernandez et al., 2014). Additionally, the *de novo* synthesis of PC_3 in roots was, like the increase in GSH, not accompanied by increases of the enzyme phytochelatin synthase *PCS* or *PCS-L* transcripts (**Fig. 5.3A**). A possible explanation could be that the regulation of PC synthesis occurs mainly post-translationally by activating the enzyme when a metal is bound to it and that regulation at the transcriptional level is of minor importance (Cobbett, 2000).

In plants under TE-induced stress, metallothioneins participate in (1) maintaining TE homeostasis, (2) sequestration of toxic metal(loid)s by forming complexes and binding to other amino acids, and (3) protection against intracellular oxidative stress (Sharma et al., 2016). The increase in *MT1A* transcripts in roots (**Fig. 5.3A**) and, more intensely, in leaves (**Table 5.12**) agrees with the fact that metallothioneins play a major role in *S. atrocinerea* metal homeostasis (Grennan, 2011).

Also in leaves, an up-regulation of aquaporins (*SIP1*, *NIP1*) was observed (**Fig. 5.4A**), suggesting a TE tolerance mechanism based on transportation across cell membranes. Phosphate transporters were also up-regulated in leaves, which could be the consequence of phosphate deprivation due to arsenate competition, as they are chemical analogues (Spratlen et al., 2017). However, P concentrations in leaves of plants grown in polluted soil were in the order of 600 mg kg^{-1} higher than in non-exposed plants (**Table 5.3**), suggesting that P could accumulate to cope with As toxicity or simply be higher as a consequence of higher P in the polluted soil. Once TEs are taken up by the plant they can be complexed by NPTs to limit their toxicity and these complexes could be transported by ABC transporters and stored in the vacuole.

Like in roots, also in leaves tyrosine phosphatase *CD25-1* was down-regulated, but *CDC25-2* was up-regulated (**Fig. 5.4A**), suggesting a role for this putative arsenate reductase in TE tolerance. The up-regulation of glutathione S-transferase (*GST*) in leaves (**Fig. 5.4A**) suggests a role for this enzyme in TE tolerance, as GST catalyzes the conjugation of GSH with metal ions and helps to sequester them into the vacuole (Yadav. 2010). The up-regulation of phytochelatin synthase *PCS* transcripts (**Fig. 5.4A**), together with the NPTs increase and the *de novo* synthesis of PC_2 in leaves of plants grown on polluted soil (**Table 5.10**), agrees with Yanitch et al. (2017), who suggested complexation by PCs to be a mechanism used in *Salix* for TE tolerance. The absence of PC_3 in leaves of field grown plants (**Table 5.11**) and the fact that this compound seems to be present constitutively as seen in leaves of plants growing on control soil, could be due to the fact that these NPTs were under the detection

limit. It is remarkable that NPTs concentrations in leaves were always higher than in roots, despite the higher TE concentrations in roots (except for Zn). In hydroponically or *in vitro* grown *S. atrocinerea* we found higher NPTs concentrations in roots, which was also the organ with the highest metal concentrations (**Chapter 3** and **Chapter 4**). A similar trend was observed with *in vitro* culture (Mesa et al., 2017) and pot grown birch (Fernandez-Fuego et al., 2017). We do not have a clear explanation for this apparent contradiction, that might be due to the different nature of the samples (Fernandez-Fuego et al., 2017): plants from pot experiments are older and have more secondary tissues than those grown *in vitro*, they present a higher proportion of lignified and suberized cells, which are less physiologically active (Ye, 2002) and, therefore, can cause a bias in the determination of concentrations as fresh weight.

The higher Cys concentrations in the leaves of plants grown in the polluted soil as compared to control plants (**Table 5.10**) can be explained by this compound being the main sulfur donor in the biosynthesis of sulfur-rich compounds such as methionine, GSH or stress-related proteins involved in the chelating response (Na and Salt, 2011; Zagorchev et al., 2013). On the other hand, since the concentrations of the most abundant toxic elements in the soil, As and Pb, were higher in leaves of plants growing in pots than from the field, it is not surprising that NTPs concentrations were higher in leaves from pot grown plants, while leaves of field grown plants showed lower PC_2, PC_4, PC_6 and TC_2, including also lower concentrations of other thiol compounds different from those observed in pot grown plants. Nevertheless, based on changes in stress indicators and gene expression, the tolerance of willow to the high concentrations of TEs accumulated in its tissues could not only be related to the differences observed in the concentrations of NPTs. Therefore, we suggest, as proposed by other authors (Zagorchev et al., 2013; Fernandez et al., 2014), that the synthesis of NPTs would not be the main mechanism for long-term TE detoxification in willow plants exposed to chronic TE stress, due to its high energy cost.

The up-regulation of *MT1A* in leaves suggests a role for metallothioneins in TE tolerance in both leaves and roots and the high up-regulation of *WBABCT*, suggests that *S. atrocinerea* leaves rely on vacuolar transporters as detoxification mechanisms. Furthermore, our transcriptomic data showing an up-regulation for the phenylpropanoid and flavonoid pathway are in accordance with Yanitch et al. (2017), suggesting, as postulated there, a role for these compounds in *Salix* to alleviate cellular toxicity.

Conceivably, inoculation can affect gene expression in *S. atrocinerea* in two ways: (1) due to the effect of the inoculation *per se* (supposedly causing similar trends between plants grown on control and polluted soils) and (2) changes due to the effect of bacterial inoculation on TE availability and speciation (that are expected to be observed only in plants

grown on the polluted soil). In roots of plants growing in polluted soil in pots, the up-regulation of *GR* in MP and MR as compared to non-inoculated plants (**Fig. 5.3C**) indicates a higher amount of GSH and enhanced capacity of defense against oxidative stress in inoculated plants. The observed up-regulation of transcripts for *PS-L* in roots of inoculated plants growing in control soil is peculiar, since PCs concentrations were below the detection limit (**Table 5.10**). Anyway, it is obvious that bacterial inoculation can influence thiol metabolism. As a consequence, there were not only differences in GSH concentrations between roots of plants growing on different soils types but also among the different treatments. Furthermore, evidence exists for the potential of microbes to enhance plant sulfur assimilation and increase their defense against herbivory (Aziz et al, 2016). Therefore, from a phytoremediation point of view it is interesting that plants inoculated with *Pantoea sp.* showed the highest NPT concentration whether on control or polluted soil.

The ATP-binding cassette (ABC) transporters are encoded by a large gene family in plants and little is known about their actual functions (Ofori et al., 2018), although their role in vacuolar As sequestration and other TEs via phytochelatin complexation has been reported (Song et al., 2010; Park et al., 2012). It has also been demonstrated that loss of a ABC transporter in *A. thaliana* results in Cd^{2+} hypersensitivity (Kim et al., 2007) as well as enhanced penetration success of pathogenic fungi (Stein et al., 2006). Therefore, these ABC proteins are likely to transport a very broad range of molecules that can be involved in pathogen responses and in the transport of molecules that mediate pathogen resistance (Kim et al., 2007). In this way, while the up-regulation of vacuolar transport transcripts of *WBABCT* (**Table 5.12**) in leaves of TE-exposed non-inoculated *S. atrocinerea* highlights its role in metal accumulation and tolerance, the up-regulation of both ABC proteins, *WBABCT* and *ABCG2*, in roots of inoculated *Salix* growing in control substrate (**Fig. 5.3B**) also suggest a microbial influence on these transporters. It is possible that *WBABCT* expression is mainly induced by the presence of TEs and to a lesser extent by bacterial inoculation. Furthermore, inoculation could lead to a biofilm on the roots of the plants and, by increasing bacterial cell concentrations or through production of exopolysaccharides, alter osmolytes concentrations at the root level and affect the water potential changing water transport in the plant and thus, vacuolar regulation (Sandhya et al., 2009).

The P concentrations in leaves of inoculated plants growing in both control and polluted soil were lower as compared to non-inoculated plants (**Table 5.3**). Therefore, inoculation with P-solubilizing bacteria *Pantoea* sp. and *R. erythropolis* was not translated into an increased P content in tissues and, in fact, bacterial inoculation caused a decrease in total P in both roots and leaves, which in the latter organ was reflected at the gene expression level by the phosphate transporter (*PHO1*) transcript down-regulation (**Table**

5.12). Although the phytochelatin synthase *PCS* was up-regulated in leaves of plants exposed to TEs (M) (**Fig. 5.4A**), a down-regulation of *PCS* after inoculation for both soil types does not correspond with the NPTs concentrations observed, highlighting again that PCS enzyme is expressed constitutively at relatively high levels regardless of nutrient status and generally unaffected by TEs (Rea et al., 2004). Like for *VAC2* and *WBABCT*, the down-regulation of *GST* and *MT1A* transcripts in leaves of TE-exposed plants (**Table 5.12**), could be related with the lower TE concentrations in these organs in MR and MP plants (**Table 5.3**). After bacterial inoculation, the phenylpropanoid and flavonoid pathways tended to be up-regulated under control conditions and down-regulated under contamination exposure when compared to non-inoculated plants in each soil type (**Table 5.12**). These results seem to be contradictory, as in both soil types condensed tannins were higher in inoculated plants, total phenols remained unaltered and only in MR plants a decrease in total flavonoids was observed (**Fig. 5.2**). All this suggests a more complicated regulation for these molecules. Nonetheless, this dual regulatory behavior might be related to the fact that flavonoids have been shown as crucial signaling molecules in the symbiosis between plants and bacteria (Liu and Murray, 2016). Phenols could play an important role in defense against excessive concentrations of toxic TEs (Wozniak et al., 2017). The differences observed for different phenolic compounds between the control and polluted soil corroborate that not only presence of TEs, but also rhizosphere and endosphere microbial communities impact phenolic signaling (Mandal et al., 2010).

Based on transcript expression, a potential role for tannins in mitigation of TEs stress in *Salix*, has been suggested by Yanitch et al. (2017) in *S. purpurea* and also by our own results (**Chapter 3**). Flavonoids have also been hypothesized to play a role in Cu, Zn, Co and aluminum (Al) chelation (McDonald et al., 1996; Chung et al., 1998; Winkel-Shirley, 2002). However, in this chapter as in **Chapter 4**, our results in *S. atrocinerea* show that at the molecular level flavonoid content is not influenced by exposure to TEs but by bacterial inoculation (**Fig. 5.2**).This high concentrations of condensed tannins after bacterial inoculation could also indicate a cross-tolerance of pollutant-exposed *Salix* against arthropod herbivory stress; increased leaf tannin concentrations could, besides enhance TE tolerance, confer the plant an advantage by reducing arthropod predation in field conditions (Gonzalez et al., 2015).

In the field, bacterial inoculation caused higher TE accumulation (**Table 5.5**), and the responses in gene expression were more similar to those observed in non-inoculated plants in the pot experiment (M), where the TE concentrations were the highest. Therefore, up-regulation of *HAPO4*, *SILICON*, *GS*, *GR*, *MT*; *WBABCT*, *ABCG2, ACCS*, *ER* and down-regulation of *CSA* are not surprising (**Fig. 5.5 and Table 5.12**). Finally, as observed in non-inoculated

plants in pots, as a consequence of higher TE concentrations, transcripts encoding enzymes regulating the phenylpropanoid and flavonoid pathways were upregulated in FP and FR (**Table 5.12**).

5.5 CONCLUSIONS

Salix atrocinerea is tolerant autochthonous species to trace elements (TEs) that grows spontaneously in the polluted soil of this study and generates a high biomass yield, being a very suitable species to use in phytoremediation processes. The contribution of the plant-associated microbial populations (rhizosphere and endosphere) is crucial in phytoremediation, and the knowledge of plant-bacteria interactions is of great practical value to improve the efficiency of phytoextraction.

Bioaugmentation by inoculation with each of the two selected endophytic (*Pantoea* sp.) and rhizospheric (*Rhodococcus erythropolis*) bacteria decreased TE accumulation in roots and leaves of *S. atrocinerea* potted plants in polluted soil. Consequently, bacterial inoculation increased the stress tolerance and plant biomass. By contrast, bacterial inoculation in the field resulted into a higher As and Pb accumulation in the plant and also a greater trace element phytoextraction, together with a markedly high Zn translocation. Regarding defense mechanisms, the role of phytochelatins in mitigating TE toxicity inside the cells was accompanied by a differential expression of genes involved in the entry of TEs into the roots, thiol metabolism and storage in vacuoles. In addition, the bacteria selected have shown a great potential to modify this process by transforming and detoxifying environmental pollutants like As, as well as to induce accumulation of certain molecules of interest in plants like tannins, emphasizing the biocatalyst potential of the plant-associated microorganisms.

Finally, extrapolation from laboratory-controlled experiments to field conditions is not a straightforward approach. Differences to inoculation response between pot and the field may be attributed to unpredictable factors such as the root exudation from host plants, inoculant losses due to leaching, pH, and/or the amount or types of microbial metabolites and soil nutrients in different settings. However, the biochemical and molecular genetic approaches adopted in this work provide a reliable basis for the rational design in the laboratory and the subsequent implementation in the field. Furthermore, it is also highlighted the necessity to improve our understanding about the mechanisms involved in TE tolerance and accumulation in plants to help to design effective microbial-assisted phytoremediation programs.

Chapter 6

Inoculation of *Arabidopsis thaliana* with *Pantoea* sp. alters genotype-specific responses to arsenic

6 Inoculation of *Arabidopsis thaliana* with *Pantoea* sp. alters genotype-specific responses to arsenic

6.1 INTRODUCTION

Arsenic (As) is a toxic metalloid found ubiquitously in the environment and widely considered an acute poison and carcinogen. Anthropogenic activities have contributed to its mobilization (e.g. combustion of fossil fuels, mining of mineral resources, production of pesticides and fertilizers) with concentrations exceeding legal toxicity values (Naidu et al., 2006). Arsenic is mostly found in its toxic inorganic forms arsenite (As III) or arsenate (As V). The former is well known for its greater mobility and toxicity relative to the latter (Tsai et al., 2009). Worldwide, As contamination is found not only in drinking water but also in various foodstuffs from animal or plant origin. Irrigation of agricultural soils with As-contaminated groundwater, especially in South-East Asian countries, contributes greatly to the accumulation of As in plants. As such, transfer of As into the food chain is a risk to human health and ecological systems (Tuli et al., 2010).

Soil remediation methods for As contamination include containment, solidification, and stabilization; however, these require appropriate controls and long-term monitoring because As is retained in the treated soil and continues to pose a leaching risk (Chang et al., 2012). Soil washing techniques using chemical agents have also been developed for As, but these involve the risk of depleting valuable minerals from the soil (Tokunaga and Hakuta, 2002). Consequently, a cost-effective remediation method that readily reduces the environmental risk posed by As with less damage to the soil is needed. To cope with this problem, a great deal of effort has been made to study the mechanisms of As mineralization by microorganisms (Kuffner et al., 2010, Das et al., 2016). Recent advances in bioinformatics and microbiological, genetic, molecular and protein engineering tools have led to several non-conventional bioremediation methods that grant a better understanding of the mode of action against As toxicity under various environmental conditions (Hernández-Prieto et al., 2014). Although a great deal of research has reported on the quantitative detection of functional genes in different bacterial systems, reports on As V reductase gene expression in bacteria remain scarce (Govarthanan et al., 2015). Hence, bacterial strain genome sequencing will allow a better characterization of the molecular mechanisms of As reduction (Wang et al., 2016).

What we know so far is that bacteria may easily alter the oxidation state of As in the soil. Some strains are able to oxidize As III to As V (Corsini et al., 2010), whereas others can reduce As V to As III, which is extruded from the cell or sequestered into the intracellular

compartment either as free As III or as conjugates with cellular thiols (Messens and Silver, 2006; Newton et al., 2008). In addition, As resistance genes are found in numerous gram-positive and -negative bacteria. They are located in the chromosome or in the plasmids and organized in an *ars* operon (Rosen, 2002; Wang et al., 2016; Fekih et al., 2018). In prokaryotic organisms, the three gene operon *arsRBC* is the most common type encoding a self-repressed transcriptional regulator (*arsR*), a membrane-bound transporter that extrudes As III out of the cell (*arsB*), and a cytoplasmic As V reductase (*arsC*) (Rosen, 2002).

The current manuscript focuses on the genus *Pantoea* as a potential tool to enhance As remediation from the soil. However, this genus comprises a number of plant pathogens (Deletoile et al., 2009). Generally, the pathogenicity of *Pantoea* sp. has been the major focus of research but in 2013, Wu et al. reported that *Pantoea* sp. IMH was the only bacterium found in this genus with a high As resistance and the first described to reduce As V to the more toxic As III. More recently, Wang et al. (2016) revealed the organization, function, and evolution of *ars* genes in two *ars* clusters (*arsR1B1C1H1* and *arsR2B2C2H2)* with different expression patterns at different temperatures. These findings highlight how bacteria might present different genetic As-resistance systems allowing the colonization of changing ecosystems, and the flexible adaptation of microorganisms to resist As. Furthermore, these bacterial interactions with As can enhance As accumulation by plants and influence the time required for soil depollution (Drewniak and Sklodowska, 2013). Apart from the previously described mechanism, bacterial metabolites such as siderophores, auxins, ethylene blockers or phosphate (P_i) solubilizers can enhance plant growth and biomass under pollutants exposure and therefore, can increase contaminant accumulation and favor contaminant removal by the plant and accelerate the phytoremediation process (Luo et al., 2011). Interesting in this regard, some bacteria possess phytases, a specific group of phosphatases capable of hydrolyzing phytate and releasing organic P into the environment. Overall, phytate is generally considered a rich reservoir of soil P and can potentially be used by soil microbes and plants (Suleimanova, 2015). This is of particular interest due to the chemical similarity between As V and P_i. Hence, bacterial inoculation of plants renders bioremediation an effective and potent approach to remove As contamination (Janssen et al., 2015; Mesa et al., 2017).

Although plants can accumulate As up to high concentrations, the molecular mechanisms of plant responses and tolerance to As have not been extensively characterized (Abercrombie et al., 2008; Finnegan and Chen, 2012). It is known that As III has an affinity for thiol groups on cysteine residues, causing deleterious effects on protein metabolism, hence its high toxicity in plants (Rai et al., 2011). In aerobic soils, As V is the most stable form of As, exerting its toxicity as an analogue of P_i by replacing it in any reaction with P_i or

a P_i-ester as a substrate (e.g. ATP synthesis) (Ali et al., 2012). Hence, potential As V-sensitive reactions include those central to the cellular metabolism (e.g. glycolysis, oxidative phosphorylation), biosynthesis (e.g. phospholipid metabolism), genome integrity (DNA/RNA metabolism), and cellular signaling (e.g. protein phosphorylation or dephosphorylation) (Finnegan and Chen, 2012). Under this dual nature, As has been shown to repress genes induced by P_i starvation, induce genes involved in oxidative stress (Abercrombie et al., 2008) and stimulate the generation of reactive oxygen species (ROS) with proven damage on proteins, lipids, and DNA (Finnegan and Cheng, 2012). In non-hyperaccumulating plant species, general tolerance mechanisms include decreased As uptake and its extrusion from cells (Murota et al., 2012). Nonetheless, once inside the cell, and despite As III being more toxic than As V, reduction to As III is a general mechanism of detoxification and tolerance in plants. Next, As III is inactivated due to its binding capacity to different intracellular chelating proteins or peptides containing thiol ligands [e.g. glutathione (GSH), phytochelatins (PCs) and metallothioneins (MTs)] and further sequestration into the plant vacuoles (Briat, 2010). Among those ligands, GSH is well known for its role as a major reservoir of non-protein thiols (Noctor, 2006). It has been shown that sufficient GSH concentrations are important in both As V reduction and As III transport and sequestration into the vacuoles (Wysocki et al., 2003).

In this study, we investigate As sensitivity and the effect of bacterial inoculation with *Pantoea* sp. on both wild-type (WT) and GSH-deficient (*cad2-1*) *A. thaliana* plants under control conditions and upon exposure to general environmental As concentrations. Observations are discussed in relation to P and As accumulation and As speciation in plant tissues. In addition, transcriptional changes are studied in detail. Understanding the mechanisms underlying decreased As sensitivity is important in directing future efforts on As stress mitigation in specific plant-based approaches.

6.2   MATERIAL AND METHODS

6.2.1   *Pantoea sp. AV62 genome sequencing, genome annotation and analysis*

RNA-free DNA was extracted from *Pantoea* sp. AV62 stationary phase cells grown in LB prior to digesting and ligating sequencing adaptors and barcodes using an Ion Xpress Plus Fragment Library Kit (Life Technologies Inc., Burlington, ON). Adaptor-ligated DNA was size selected to 580 bp on a 2% E-Gel SizeSelect agarose gel, and Agencourt MAPure XP beads (Beckman Coulter, Mississauga, ON) were used for purification. The library dilution factor was determined using an Ion Library Quantitation Kit prior to amplification with an

Ion PGM Template IA 500 Kit and enrichment with an Ion One Touch Enrichment System. Sequencing was performed on a 316v2 chip with an Ion PGM Hi-Q View Sequencing Kit on an Ion Torrent PGM (Life Technologies Inc., Carlsbad, CA).

1.07 million reads (mean length 319 bases) generated 341 Mb of data (> 311 M Q20 bases) in Torrent Suite 5.0.5. These were assembled using SPAdes 3.8.2 (Bankevich et al., 2012; Gurevich et al., 2013) (uniform coverage mode; kmers 21, 33, 55, 77, 99). 57 contigs greater than 1000 bp, giving a consensus length of 4 973 366 bp at 40 X coverage (GC content 55.77%; largest contig 605 363 bp; N50 = 195 579 bp). The RAST Server (Aziz et al., 2008, Overbeek et al., 2014; Brettin et al., 2015) indicated that closely related strains are *3.5.1 and UBA5037*. The genome from RefSeq was used as a reference to order the contigs using MAUVE. ORF prediction and gene annotation completed using the NCBI PGAP pipeline (Angiuoli et al., 2008). This strain has a GC content of 56.1 % and 4.739 genes. The draft genome has been deposited in GenBank and the accession number used is GCA_002233725.1

6.2.2   Selection and characterization of Pantoea sp. AV62 resistance to As

The phosphorus solubilizing, nitrogen-fixing, indole-3-acetic acid (IAA)-, 1-aminocyclopropane-1-carboxylate (ACC) deaminase- and siderophore-producing *Pantoea* sp. AV62 was previously selected from the endosphere of a *Salix atrocinerea* growing in an As-brownfield (**Chapter 2**). In addition, *Pantoea* sp. AV62 was able to resist up to 150 mM AsV and 20 mM As III in 284 medium (Schlegel et al.,1961).

In order to investigate the transcription levels of each gene in the *ars17* and *ars19* clusters under differential environmental factors, RTqPCR analysis was performed. *Pantoea* sp. AV62 was grown in 869 medium (Mergeay et al., 1985) with 1 mM As V as $Na_2HAsO_4.7H_2O$ at different temperatures (15 °C and 30 °C) for 24 h. Temperature selection was based on a previous study by Wang et al. (2016) who reported differential *ars* operon induction at those temperatures. Next, the cDNA was obtained as described in the gene expression analysis. Specific cDNA was used to quantify the transcriptional signals of the *ars* clusters and *arsC* genes, where 16S rRNA was used as an internal reference. Primers used are listed in ***Supplementary Table* 2**.

6.2.3   Plant and bacterial growth

Arabidopsis thaliana ecotype Col-0 (WT) seeds were obtained from NASC (Nottingham Arabidopsis Stock Centre) and mutant *cad2-1* was provided by Dr Christopher Cobbett (Melbourne University, Australia). This mutant was selected in a screen for Cd sensitivity and carries a mutation in the GSH biosynthesis gene γ-glutamylcysteine

synthetase (*GSH1*, *AT4G23100*), resulting in approx. 15% – 40% of WT GSH levels (Howden et al., 1995; Cobbett *et al.*, 1998).

Seeds were surface sterilized in 0.1% (w/v) NaOCl and 0.1% (v/v) Tween 80 for 1 min and washed four times with sterile water during 20 min. After sterilization, they were cultivated on 12x12 cm vertical agar plates (VAPs) containing ¼ Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 5 g L^{-1} sucrose, and incubated at 4°C in the dark for 2 days to ensure homogeneous germination. Germination plates were then set up vertically in a growth chamber at 22/18 °C (day/night) and 12h light per day (150 μmol m^{-2} s^{-1} at leaf level). After 7 days of growth, plants were transferred to treatment plates containing ¼ MS medium and supplemented with 0 or 70 μM As V in the form of Na$_2$HAsO$_4$.7H$_2$O and inoculated with or without *Pantoea* sp. AV62. The As dose was chosen according to the tolerance shown by *cad2-1* in the presence of increasing doses of As V under *in vitro* conditions (data not shown). The top 1 cm of the agar of the exposure plates was cut off so the leaves were not in contact with the medium and the metal exposure occurred solely via the roots.

Pantoea sp. AV62 was isolated from the endosphere of roots of *Salix atrocinerea* growing in an As-polluted soil and tested for As V reduction and plant-growth promoting characteristics (IAA production, ACC-deaminase activity, P solubilization, As V reductase and production of siderophores, organic acids and acetoin) as described in **Chapter 2**. For inoculation, bacteria were grown overnight in rich 869 medium (Eevers et al., 2015) and harvested in the late exponential phase. The cultures were centrifuged (3000 *g*, 30 min) and the pellets were resuspended in 10 mM MgSO$_4$ until 10^4 cfu mL^{-1} was reached. Next, 100 mL bacteria solution was spread on the growth plates.

After 10 days of growth on treatment plates (0 or 70 μM As) in the presence or absence of bacteria, roots were untangled to improve analysis and the plates were scanned at 300 dpi using an Epson flatbed scanner. The root images were analyzed using the RootNav software (University of Nottingham)

6.2.4 *Analysis of the concentrations of phosphorous, arsenic and arsenic speciation in plant tissues*

The concentrations of the macronutrient P together with As, were determined in shoots and roots of *A. thaliana*. For this, 100 mg of dry powdered and processed as described in ***section 2.3.1.***

To determine the As speciation in shoots and roots, 100 mg of finely-ground sample in 2.5 mL of 0.3 M nitric acid solution and analyzed as described in ***section 2.3.1.***

6.2.5 Gene expression analysis in plants and bacteria

Frozen leaf samples were shredded twice in liquid nitrogen-cooled adapters for 1 min at 30 s^{-1} frequency (Retsch Mixer Mill MM400, Verder Scientific GmbH & Co. KG, Haan, Germany). Next, RNA was extracted from the homogenized samples using the Ambion™ RNAqueous® Kit (Life Technologies, Waltham, MA, USA) and eluted in RNase-free water pre-heated at 80 °C. Concentration and quality of the RNA samples were verified using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., MA, USA). Prior to cDNA synthesis, the RNA samples were cleaned of genomic DNA using the TURBO DNA-free™ Kit (Life Technologies). Complementary DNA was synthesized from equal amounts (1.1 μg) of cleaned RNA samples using the PrimeScript™ RT Reagent Kit (Perfect Real Time) (TAKARA BIO Inc., Shiga, Japan) and the thermal cycler Techne TC-5000 (Life Technologies). The cDNA samples were diluted in 1/10 TE (Tris-EDTA) buffer (1 mM Tris-HCl, EDTA 0.1 mM, pH 8.0) and stored at −20 °C.

RTqPCR was performed using the Applied Biosystems™ Fast SYBR® Master Mix (Thermo Fisher Scientific, Inc.) and 300 nM, unless stated otherwise, of gene-specific forward and reverse primers (**Supplementary Table 3**). Primer efficiencies were determined using a standard curve consisting of a two-fold dilution series of a pooled sample. **Supplementary Table 3** shows the qPCR parameters according to the MIQE guidelines (Bustin et al., 2009). After an initial denaturation at 95 °C for 20 s, the amplification reaction involved 40 cycles of denaturation at 95 °C for 3 s followed by annealing/elongation at 60 °C for 30 s and was performed in the Applied Biosystems™ 7500 Fast Real Time PCR System (Life Technologies). Subsequently, a melting curve was generated to verify amplification specificity. After analyzing 10 candidate reference genes (Remans et al., 2008), *ACT*, *TIP41*, *UBC* and *UBC4*, were selected using the GrayNorm algorithm (Remans et al., 2014) to normalize the expression levels of the genes of interest (**Supplementary Table 3**). Expression levels were determined for several genes of interest concerning the As metabolism in shoot samples.

Hierarchical clustering analysis was performed (GenEx software, v6, MultiD Analyses AB, Sweden) to recognize potential sample-related patterns during As exposure in two *A. thaliana* genotypes (WT and *cad2-1*). The analysis was based on raw gene expression values and the 'Average linkage' algorithm, defining the distance between conditions as the average of distances between all pairs of individuals in all groups. Distances between the measures were calculated via the Euclidian Distance Measure. Heat maps were constructed to compare expression levels between different genes and samples.

Bacterial presence in *A. thaliana* shoots was determined by relative expression of 16S rRNA's bacterial transcripts, for which primers were designed to avoid unspecific amplification of plant DNA, as well as to generate shorter PCR products appropriate for qPCR as described above. The qPCR of 16S rRNA gene was also performed in the same way as previously described.

6.2.6   *Statistical analysis*

To evaluate the effects of As toxicity in *A. thaliana* in the different treatments on the measured variables, a two-way Analysis of variance (ANOVA) was performed. Data were treated and transformed when necessary as described in **section 3.2.5**. In the gene analysis the previous was performed on both the normalized and the non-normalized data, although only the first are presented both were taking into account to establish the significance of the results. Results are expressed as the mean ± standard deviation of at least three independent replicates. All data were analyzed using R (version 3.3.1, http://www.r-project.org/) with the packages mixOmics (for PCA, version 6.0.1, http://www.mixOmics.org) and agricolae (version 1.2e4, http://tarwi.lamolina.edu.pe/~fmendiburu). Outliers were determined using the Extreme Studentized Deviate method (GraphPad Software, La Jolla, CA, USA) at significance level $p \le 0.05$.

6.3 RESULTS

6.3.1 *Characterization of As resistance of Pantoea sp. AV62*

We here report the draft assembly for the genome of *Pantoea* sp. strain AV62, isolated from an As-polluted soil (Langreo, 43°18'49"N, 5°42'05"W, Asturias, Spain) (https://www.ncbi.nlm.nih.gov/Traces/wgs/?val=NHBE01). Exposure to 1mM As in the laboratory caused a decrease in bacterial growth, especially at a lower temperature (**Fig. 6.1**). At both temperatures, *Pantoea* sp. AV62 showed the ability to aerobically reduce As V to As III, with a larger reduction at higher temperatures (**Fig. 6.2**).
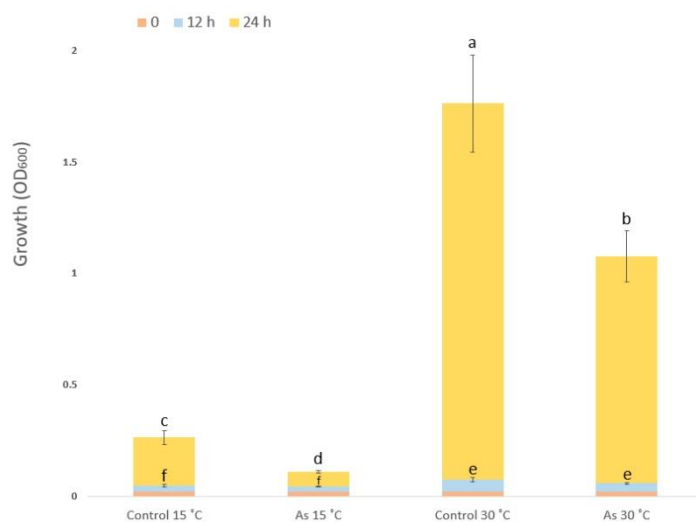
We analyzed if function-based RAST annotated genes were possibly responsible for As V reduction by reporting the transcription levels of the pertinent genes (*arsH*, *arsB*, *arsC* and *arsR*) involved in regulating As V reduction and resistance identified in the genome of *Pantoea* sp. AV62. We observed that *Pantoea* sp. AV62 has two *ars* systems - a four gene *ars17*

Fig. 6.1. Growth of *Pantoea* sp. AV62 in liquid 869 medium non-exposed and exposed to As V for 24 h at different temperatures in liquid 869 medium. Different letters denote significant differences on HSD test at $p < 0.05$.

(*arsR17B17C17H17*) and a three gene *ars19* (*arsR19B19C19*) - with low sequence homology (**Fig. 6.3**) and non-clustered arsenate reductase (*arsC8*). The genes in each cluster were separated by a short sequence of only a few nucleotides, suggesting they were organized in the same operon. On the one hand, we observed that all genes of *ars17* and *ars19* clusters were completely transcribed. Notably, *ars17* genes exhibited higher transcription levels than *ars19* genes at 30 °C, whereas this was reversed at 15 °C for most genes. On the other hand, the *arsC8* gene had a very low transcription at both temperatures tested (**Fig. 6.4**),

Fig. 6.2. Reduction of arsenate (As V) to arsenite (As III), measured as an increase in As III, by *Pantoea* sp. AV62 exposed to As V for 24 h at different temperatures in liquid 869 medium. Different letters denote significant differences on HSD test at $p < 0.05$.

indicating that this gene only has a minor contribution to As resistance. In addition, other genes responsible for As binding, tolerance and transport were also identified and measured (**Supplementary Table 2**). In general, transcript levels of genes related to the S metabolism [sulfate uptake (*cysZ*), cysteine synthase (*cys1/2*) GSH biosynthesis (*gsh*), GSH reductase (*gr*), GSH transferase (*gst*)] were higher in bacteria exposed to As V at 12 h with a significant regulation at 15 °C. However, at 24 h, a general downregulation at both temperatures was observed for all the genes measured in bacteria exposed to As V as compared to the control condition (**Fig. 6.5**). Transcript levels of glutamate-cysteine-ligase (*gcl*) were downregulated along the experiment in bacteria exposed to As V. In As-exposed conditions, *grx2* and *grx3* were upregulated after 12h at 15 °C and *grx1* was downregulated but upregulated at 30 °C as compared to controls (**Fig. 6.5**). Lower transcript levels for thioredoxins (*trx1/2*) were observed for bacteria along the As exposure period as compared to bacteria in control conditions. However, thioredoxin reductase transcripts (*trxR*) were significantly induced in As-exposed bacteria at 15 °C. A similar trend was observed for *pit1* transcripts of the Pi transporter whereas no changes were observed for *pit2*. A downregulation in transcripts for aquaporin (*aqpZ*) was observed at 24 h at both temperatures. *Pantoea* sp. AV62 phytase (*agpP*) was upregulated at both 15 and 30 °C at 12 h, whereas a downregulation in transcript levels was
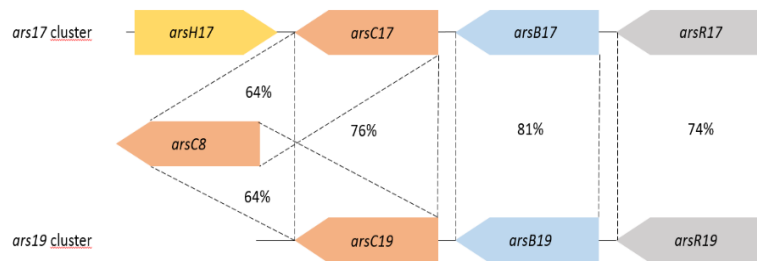
Fig. 6.3. Homology analysis of two *ars* clusters and a non-clustered arsenate reductase (*arsC*8) based on gene sequences.

observed at 24 h at both temperatures (**Fig. 6.6**).

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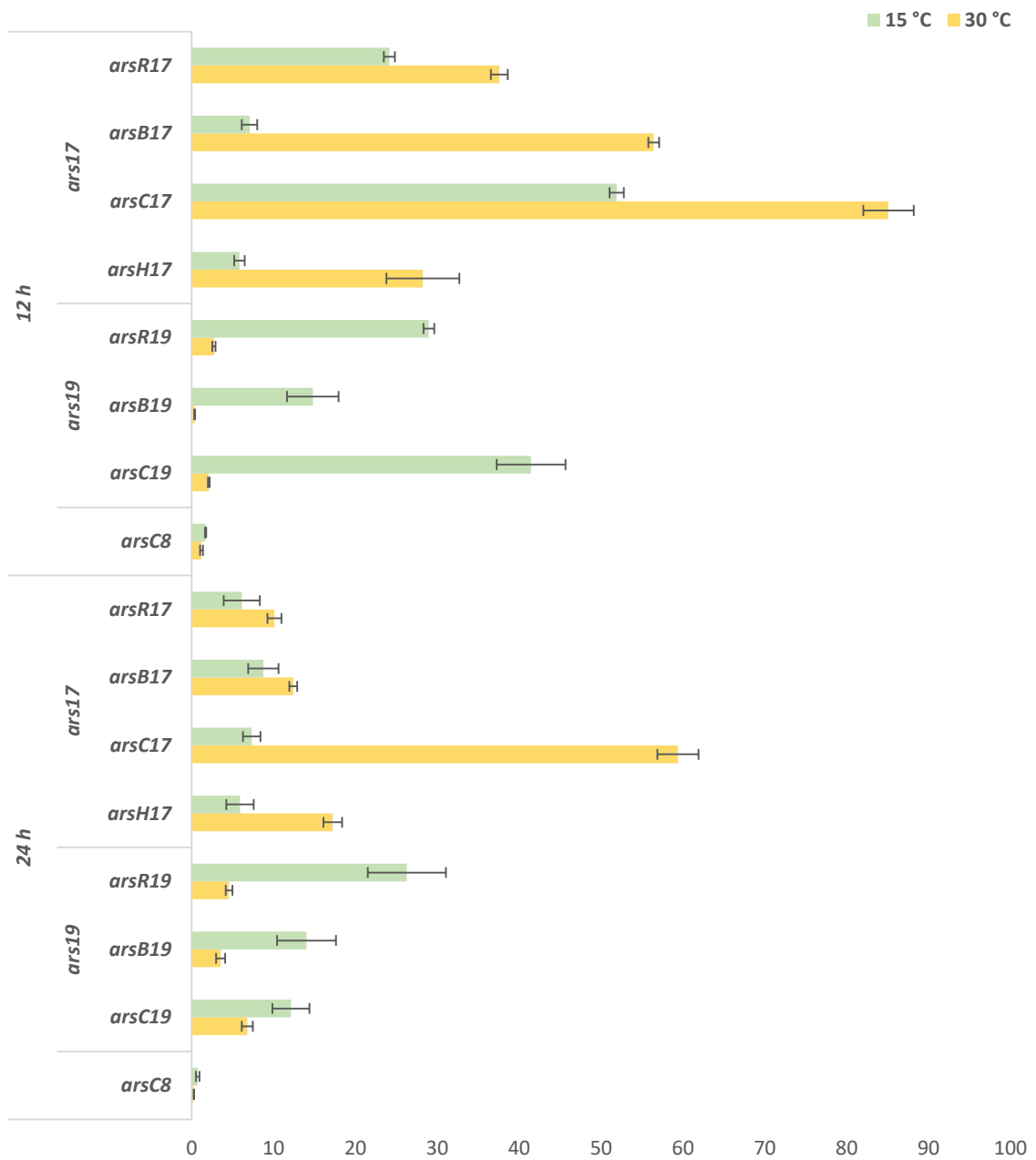
Fig. 6.4. Relative transcription level of *ars* clusters and *arsC* genes of *Pantoea* sp. AV62 exposed to As V for 24 h at different temperatures in liquid 869 medium. Values are mean normalized expression relative to the non-exposed bacteria at each time point (set at 1.00) ± S.D. of at least three biological replicates.
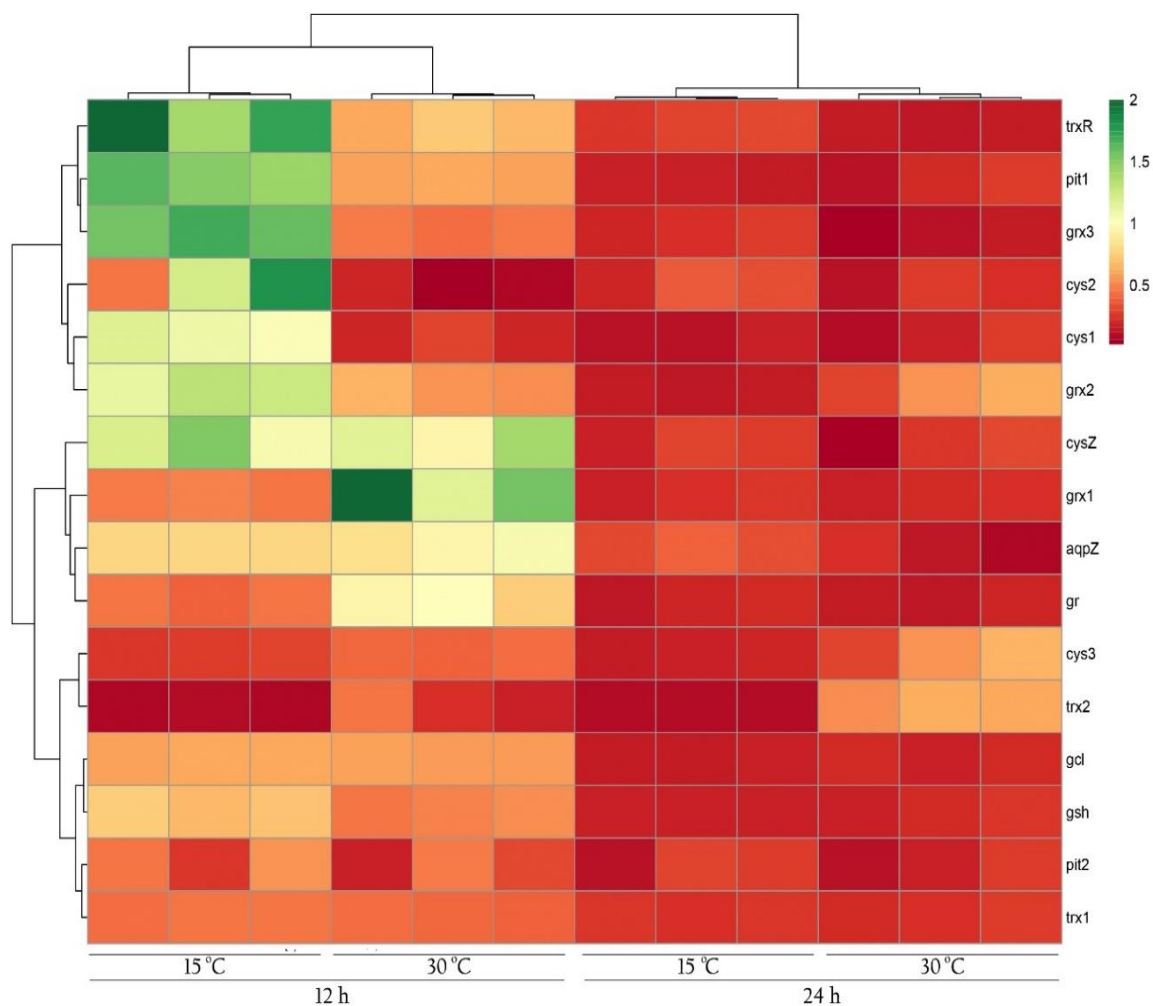
Fig. 6.5. Heat map representations of the gene expression data obtained in samples of *Pantoea* sp. AV62 exposed to As V for 24 h at different temperatures in liquid 869 medium. Hierarchical clustering based on the most differentially expressed genes. Gene expression level values are the normalized expression relative to the non-exposed bacteria at each time point and temperature of at least three biological replicates. Green-shaded rectangles indicate increased, while red-shaded rectangles indicate decreased gene expression. For gene abbreviations see **Supplementary Table 2.**
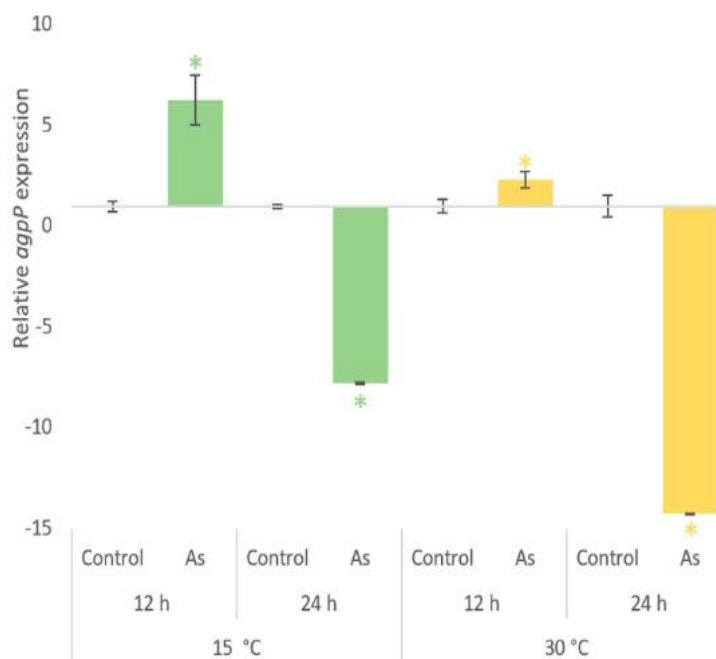
Fig. 6.6. Relative transcription level of *Pantoea* sp. AV62 phytase *agpP* exposed to As V for 24 h at different temperatures in liquid 869 medium. Values are mean normalized expression relative to the non-exposed bacteria at each time point and temperature (set at 1.00) ± S.D. of at least three biological replicates.

6.3.2   Bacterial gene expression upon inoculation in A. thaliana

Pantoea sp. AV62 inoculated on the agar plates effectively colonized shoot tissues of *A. thaliana* (**data not shown**). Therefore, gene expression of the *arsC* genes were analyzed in order to investigate whether they might contribute to the As speciation in the plants. Gene expression of the different *arsC* genes showed that in shoots, all *arsC* were downregulated in inoculated As-exposed plants 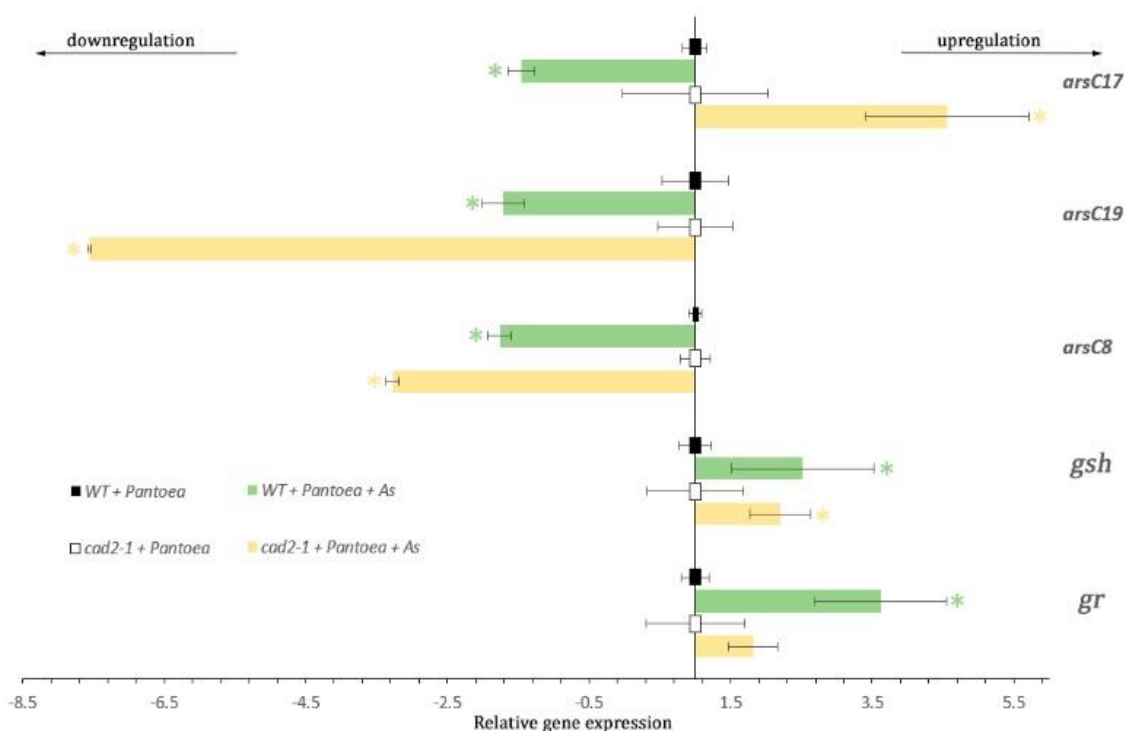as compared to control plants, with the exception of *arsC17* in *cad2-1* mutant plants, where a 4.5-fold upregulation was observed (**Fig. 6.7**). Genes involved in GSH metabolism like *gsh* was upregulated in *Pantoea* sp. AV62 when present in shoots of both genotypes exposed to As, whereas *gr* was only upregulated in WT plants (**Fig. 6.7**).

Fig. 6.7. Relative transcription level of different arsenate reductases (*arsC*) and glutathione synthase (*gsh)* and glutathione reductase (gr) genes of *Pantoea* sp. AV62 in samples of shoots of *A. thaliana* at 10 days of culture in VAPs under different treatments. Values are mean normalized expression relative to that of *Pantoea* sp. AV62 in shoots of non-exposed plants to As (set at 1.00) ± S.D. of at least three biological replicates. *Significant up or down-regulation at p < 0.05.

6.3.3 Plant growth

For an in-depth analysis of the effects in root growth, primary root length, lateral root length and lateral root number were measured. In control conditions, no difference was found between WT and *cad2-1* roots. When WT plants were inoculated, no effect on the primary root length was observed (**Fig. 6.8A**) but the lateral outgrowth (**Fig. 6.8B**) was decreased and the lateral root number was increased (**Fig. 6.8C**), regardless of As exposure. In *cad2-1* mutant roots, bacterial inoculation did not affect root growth during control conditions (**Fig. 6.8A**).

Both primary and lateral root growth of WT plants were unaffected by exposure to As (**Fig. 6.8A**). However, in the *cad2-1* mutants the primary root growth was reduced by As exposure (**Fig. 6.8A**). As exposure also inhibited lateral elongation of *cad2-1*, while stimulating the number of lateral roots and consequently their density (**Fig. 6.8C, D**).
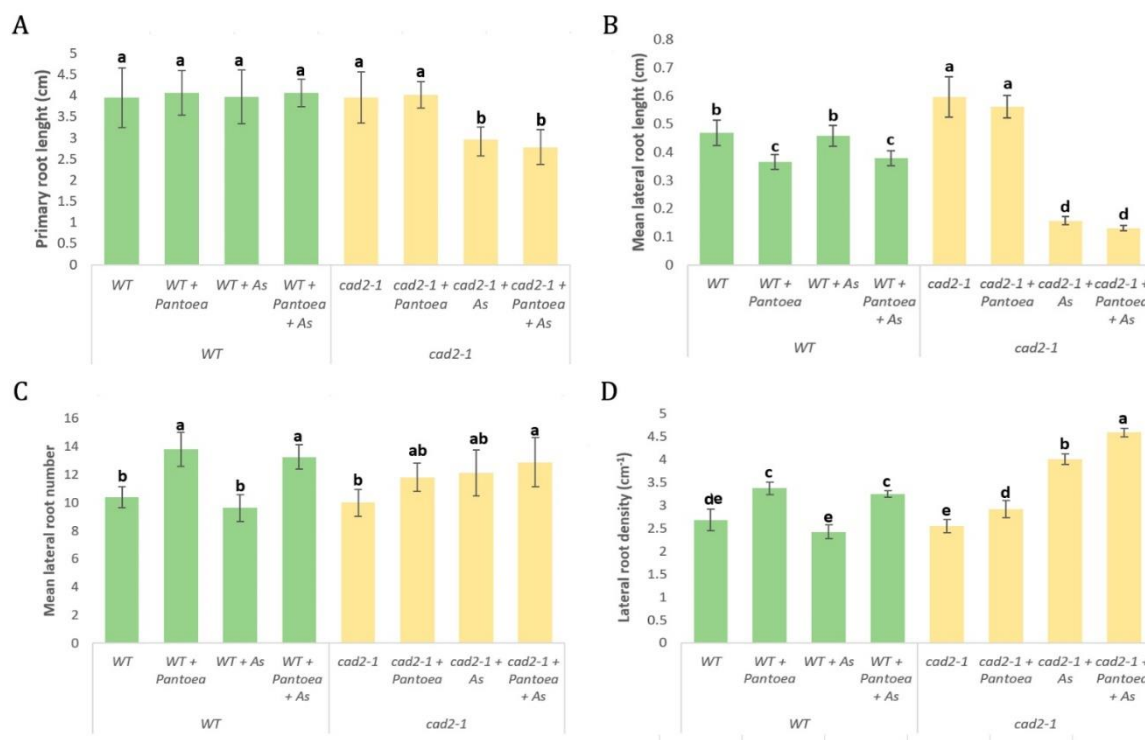
Fig. 6.8. Primary root length (A), total lateral length (B), mean lateral root length (C), mean lateral root number (D), lateral root length/unit primary root length (E) and lateral root density (F) in roots of *A. thaliana* at 10 days of culture in VAPs under different treatments. Different letters denote significant differences on HSD test at p < 0.05.

In non-exposed conditions, *cad2-1* mutant plants showed a lower shoot fresh weight than WT plants (**Fig. 6.9**). When inoculated with *Pantoea* sp., there was an increase of fresh weight in the shoots of WT plants, whereas this increase after inoculation was observed in both roots and shoots of *cad2-1* mutants as compared to non-inoculated plants (**Fig. 6.9**). When exposed to As, the fresh weight of *A. thaliana* WT and *cad2-1* mutant roots and shoots were both decreased when compared to control plants. This decrease was less pronounced in WT shoots or even resolved in WT roots when plants were inoculated with *Pantoea* sp. AV62 (**Fig. 6.9**). However, in the *cad2-1* mutant, bacterial inoculation did not reduce the growth decrease related to As exposure.
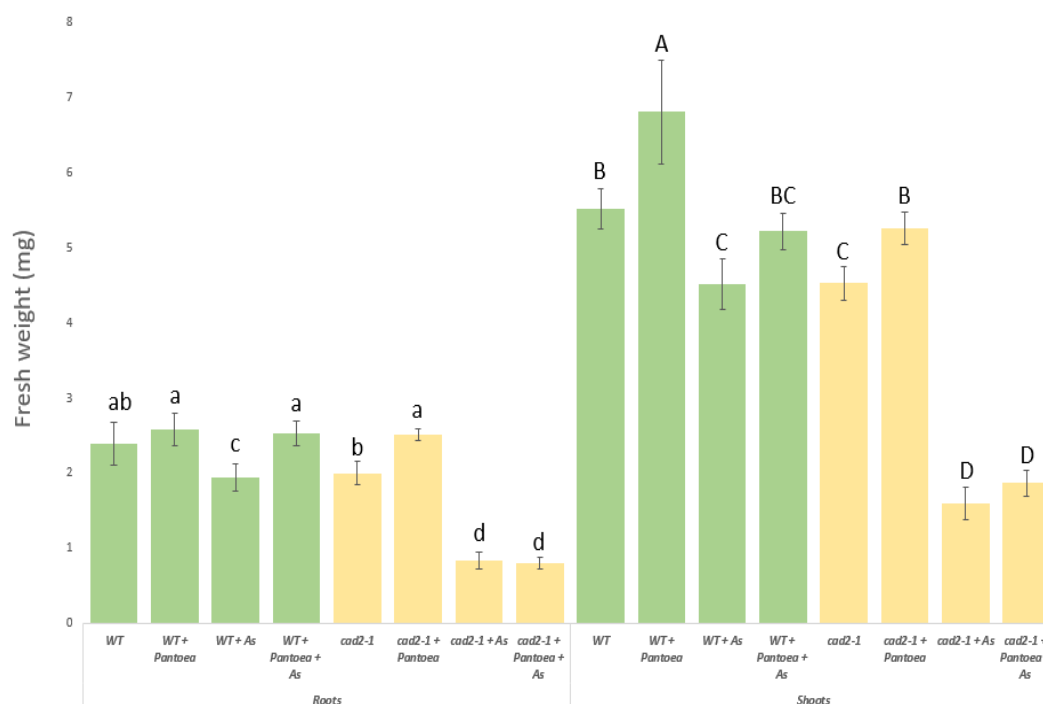
Fig. 6.9. Fresh weight of roots and shoots of *A. thaliana* at 10 days of culture in VAPs under different treatments. Different letters (upper case for comparison within roots and lower case for comparison within shoots) denote significant differences on HSD test at p < 0.05.

6.3.4   *P concentration, As accumulation and speciation in plants*

In roots of *A. thaliana* WT and *cad2-1* mutant plants, P concentrations showed no differences under the different treatments (**Table 6.1**).

In shoots, *cad2-1* mutants presented a higher P concentration than WT plants under non-exposed conditions. Whereas bacterial inoculation increased the P concentration of WT plants, no stimulation of the bacterial effect on P concentrations was observed for *cad2-1* mutants (**Table 6.1**). Exposure to As did not affect P concentrations of WT plants and bacterial inoculation increased the P concentration to the same levels as was observed

under non-exposed conditions (**Table 6.1**). However, As-exposed *cad2-1* mutant plants showed a decrease in P concentration in the shoots and this decrease was even more pronounced in inoculated As-exposed plants (**Table 6.1**).

Concerning As uptake and distribution after As exposure, roots of WT plants contained 3-fold higher As concentrations than roots of *cad2-1* mutants. A similar trend was observed in shoots where the As concentration was 2-fold higher in WT plants than in shoots of *cad2-1* mutants (**Table 6.1**). Total As levels in WT plants were increased upon inoculation with *Pantoea* sp., which was not the case in *cad2-1* plants showing the same As levels in roots and lower As levels in shoots as compared to the non-inoculated mutants (**Table 6.1**).

WT plants had a high capacity for reducing As V to As III, with 89% and 100% presence of As III of the total As in roots and shoots, respectively (**Fig. 6.10**). The capacity for As V reduction was lower in the *cad2-1* mutant, where the amount of As III was lower in both roots and shoots as compared to WT plants. Nonetheless, most of the As in roots (74%) and shoots (90%) of the mutant was reduced to As III (**Fig. 6.10**). The proportion of As III was higher in shoots than in roots in both genotypes. When plants where inoculated with *Pantoea* sp. under As exposure, there was an increase in the percentage of As III present in roots for both genotypes, with 94% and 80% of As III for WT and *cad2-1* plants, respectively. In shoots, bacterial inoculation increased the percentage of As III to 95% in the *cad2-1* mutants (**Fig. 6.10**).

Table 6.1. Phosphorous concentration (g kg^{-1} DW) and As accumulation (mg kg^{-1} DW) in roots and shoots of *A. thaliana* at 10 days of culture in VAPs under different treatments. Different letters within the same column and organ denote significant differences on HSD test at $p < 0.05$.

Treatments	Roots		Shoots		Shoot/Root	
	P	As	P	As	P	As
WT	11.04 ± 0.53 a	-	14.82 ± 1.17 c	-	1.34	0.61
WT + *Pantoea*	11.33 ± 0.47 a		17.21 ± 0.89 b	-	1.52	
WT + As	11.05 ± 0.47 a	933.99 ± 73.37 b	13.98 ± 0.02 c	88.49 ± 15.72 b	1.26	0.09
WT + *Pantoea* + As	11.12 ± 0.49 a	1422.79 ± 64.86 a	19.10 ± 0.93 ab	120.31 ± 11.83 a	1.72	0.08
cad2-1	10.14 ± 1.68 a	-	20.78 ± 1.02 a	-	2.05	0.21
cad2-1 + *Pantoea*	11.50 ± 1.23 a	-	21.50 ± 0.90 a	-	1.87	
cad2-1 + As	10.07 ± 0.76 a	284.31 ± 23.98 c	12.10 ± 1.00 c	40.71 ± 3.38 c	1.20	0.14
cad2-1 + *Pantoea* + As	12.58 ± 1.90 a	333.89 ± 29.30 c	9.14 ± 0.94 d	29.18 ± 4.53 d	0.73	0.09
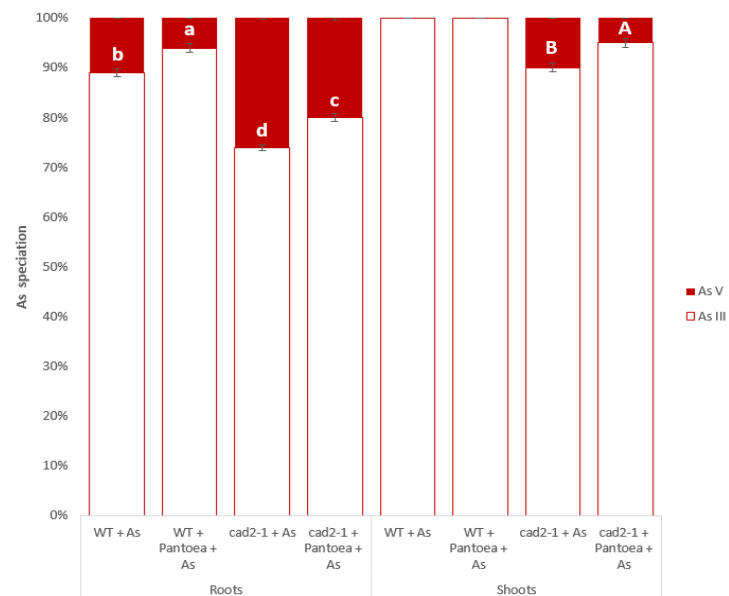
Fig. 6.10. Percentage of As III and As V in roots and shoots of *A. thaliana* at 10 days of culture in VAPs under different treatments. Different letters (upper case for comparison within roots and lower case for comparisons within shoots) denote significant differences on HSD test at $p < 0.05$.

6.3.5   Gene expression in A. thaliana

As we observed that *Pantoea* sp. AV62 was able to colonize the shoots of *A. thaliana* in our experimental setup, transcript levels of several genes were analyzed in order to determine whether inoculation with *Pantoea* sp. AV62 affected the defense responses of the plants in order to preserve acclimation to As exposure. Expression levels of several genes involved in As transport and chelation, hallmarks of oxidative stress as well as pro- and antioxidative responses, the secondary metabolism, the ethylene pathway and genes encoding transcription factors were determined in shoots of WT and *cad2-1* plants under control conditions or exposed to As with or without inoculation.

In general, we can see that gene expression of shoots of WT and *cad2-1 A. thaliana* plants grown under different treatments clustered together (**Fig. 6.11**). This indicates a clear treatment genotype-related response.

Under control conditions, gene expression data revealed the following genes being differently expressed in the shoots of both genotypes: the As-related gene *AsI3* as well as the oxidative stress-related *CAT1* and hallmark *UPOX* gene showed higher expression levels in *cad2-1* mutants relative to WT plants. An opposite expression pattern was observed for S assimilation-related genes (*SULTR1;2*, *APR1/2/3* and *GGCT1;2*), P_i-related (*ACP5* and *MGD3*), and oxidative stress hallmark (*AT1G19020* and *TIR*) genes (**Table 6.2**). With regard to genotype-related differences in gene expression under inoculation, *cad2-1*-inoculated plants differed from WT-inoculated plants with higher transcript levels of the As-related *AsI3* gene, the ethylene-related *ETR2* and stress-related *UPOX* expression and a decrease in expression levels for P_i-related genes (*ACP5* and *MGD3*), S-related genes (*SULT2;1*, *APR2/3*, *GRX480* and *GT11*), ethylene-related *ERF1* and oxidative stress hallmark genes (*AT1G19020* and *TIR*) (**Table 6.2**).

In As-exposed plants, transcript level analysis confirmed genotype-specific differences between WT and *cad2-1* plants, with a higher gene expression detected in *cad2-1* mutants for P_i-related (*ACP5*, *MGD3*), S-related (*SULTR1;2/2;*1, APR1*/2/3, GGCT1;2, GRX480*), S and As-related (*MT2a* and *AsI3/4*, *GST11* respectively) and oxidative stress-related (*CSD1/2, CAT1* and the five oxidative stress hallmarks) genes. After inoculation, the S-related (*GGCT1;2* and *GRX480*) and oxidative stress-related (*CAT1* and markers *DELF*, *AT1G19020* and *TIR*) genes showed no more pronounced changes in transcript levels in As-exposed *cad2-1*-inoculated mutants when compared to As-exposed WT-inoculated plants (**Table 6.2**).

For the other genes, a similar trend in transcript regulation was observed as previously mentioned under As exposure without inoculation. In addition, also a higher expression of the metalloid-chelating *MT1a*, ethylene-related *ETR2* as well as oxidative stress-related (*CCS* and *SOD2*) genes was observed and a lower expression of the metalloid-chelating *PCS1* and *ETR2* genes was observed in *cad2-1* plants (**Table 6.2**).

Focusing on the effect of the different treatments on each genotype, WT-inoculated plants showed a downregulation of P_i-related (*ACP5* and *MGD3*) and S-related (*APR1/3* and *GGCT1;2*) genes (**Table 6.3**) compared to WT plants without inoculation. On the one hand, exposure to As caused an upregulation of P_i transporter *PHT1;1*, ethylene-related *ETR2* and oxidative stress hallmark *UPOX* transcripts levels and a downregulation of As III transporter *NIP7;1*, S-related (*APR1/2/3*, *GGCT1;2*), P_i-related (*ACP5* and *MGD3*), and oxidative stress hallmark *TIR* genes (**Table 6.3**). Furthermore, bacterial inoculation under As presence increased transcript levels of P_i transporter *PHT1;1*, S-related genes (*PCS1*, *SULT1;2*, *APR1*, *GGCT1;2*, GT11, *GRX480*), P_i-related (*ACP5* and *MGD3*), ethylene-related (*ACS6, ERF1*), oxidative stress-related (*PX*) as well as oxidative stress hallmark (*DEFL, AT1G19020, TIR*) genes as compared to As-exposed non-inoculated treatments (**Table 6.3**).

With regard to the *cad2-1* mutant, inoculation with *Pantoea* sp. AV62 did not alter transcript levels for any of the genes measured. However, when *cad2-1* plants were exposed to As there was an upregulation for most of the genes measured, especially P_i-related (*ACP5, MGD3*), S-related (*MT2A, SULT1;2/2;1, APR1/2/3, GGCT1;2, GRX480, GT11*) and the five oxidative stress hallmark genes, whereas a downregulation was observed for *NIP7;1* as compared to non-exposed mutant plants (**Table 6.3**). Furthermore, when inoculated, a greater upregulation was observed for As-related *AsI4*, P_i-related *MGD3*, S-related (*SULT1;2/2;1, APR3, GGCT1;2, GST11*), ethylene-related (*ACS6, ERF1, WRKY33*), oxidative stress *CSD1,* as well as *DELF* and *TIR* hallmark genes relatively to the non-inoculated and As-exposed *cad2-1* plants, together with a downregulation of *FSD1* transcript levels (**Table 6.3**).
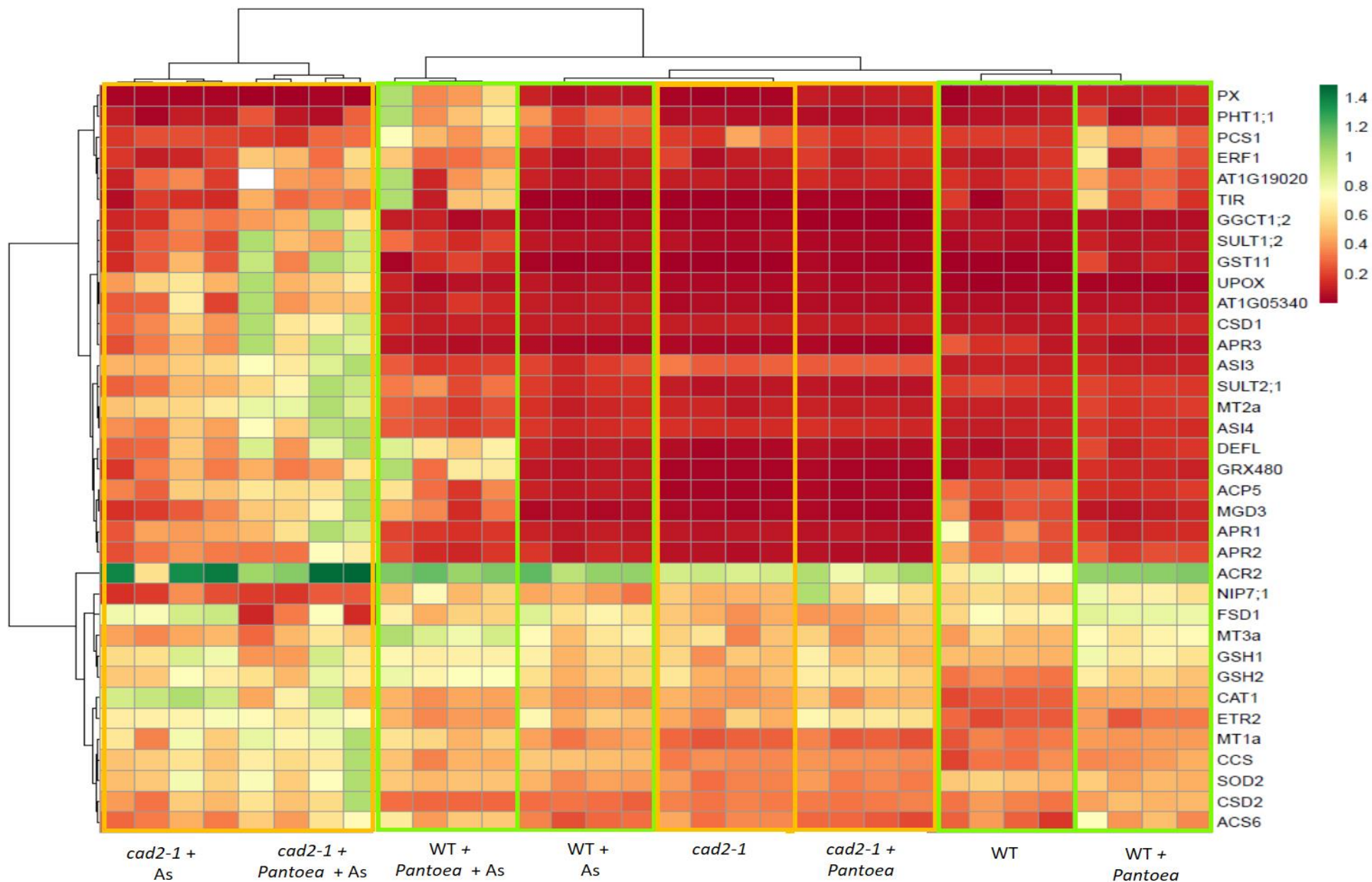
Fig. 6.11. Heat map representations of the gene expression data obtained in samples of shoots of *A. thaliana* at 10 days of culture in VAPs under different treatments and hierarchical clustering based on the most differentially expressed genes. Gene expression level values are the normalized expression relative to the non-exposed plants at each time point of at least four biological replicates, each containing at least one individual plant. Green-shaded rectangles indicate increased, while red-shaded rectangles indicate decreased gene expression. For gene abbreviations see *Supplementary Table 3*.

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Table 6.2. Relative gene expression levels obtained in samples of shoots of *A. thaliana* at 10 days of culture in VAPs under different treatments. Values are mean normalized expression relative to the indicated treatment (set at 1.00) ± S.D. of at least four biological replicates, each containing at least one individual plant. Statistically significant at p < 0.05 treatment-induced changes in expression relative to the indicated treatment are marked by color (■ = upregulation; ■ = downregulation). For gene abbreviations see ***Supplementary Table 3***.

Gene	Gene expression level relatively to			
	WT	WT + *Pantoea*	WT + As	WT + *Pantoea* + As
	cad2-1	*cad2-1* + Pantoea	*cad2-1* + As	*cad2-1* + Pantoea + As
As-related				
PHT1;1	0.66 ± 0.03	0.86 ± 0.21	0.29 ± 0.05	0.36 ± 0.12
NIP1;7	0.72 ± 0.06	1.24 ± 0.15	0.49 ± 0.05	0.46 ± 0.02
ACR2	1.20 ± 0.05	0.95 ± 0.05	1.17 ± 0.16	1.12 ± 0.10
AsI3	2.30 ± 0.09	2.74 ± 0.16	2.79 ± 0.24	4.03 ± 0.15
AsI4	1.18 ± 0.04	1.30 ± 0.11	2.47 ± 0.23	3.91 ± 0.37
Pi-related				
ACP5	0.09 ± 0.01	0.30 ± 0.03	4.11 ± 0.59	2.64 ± 0.39
MGD3	0.16 ± 0.01	0.55 ± 0.06	4.46 ± 0.75	3.05 ± 0.53
S-related				
GSH1	1.06 ± 0.13	1.03 ± 0.09	1.29 ± 0.13	0.96 ± 0.14
GSH2	1.35 ± 0.08	1.38 ± 0.12	1.17 ± 0.11	0.99 ± 0.07
PCS1	1.25 ± 0.31	0.70 ± 0.11	0.87 ± 0.03	0.43 ± 0.04
MT1a	0.85 ± 0.04	0.91 ± 0.05	1.57 ± 0.24	1.75 ± 0.16
MT2a	0.82 ± 0.05	0.72 ± 0.06	4.32 ± 0.32	4.63 ± 0.06
MT3	0.92 ± 0.06	0.84 ± 0.06	0.72 ± 0.06	0.63 ± 0.07
SUL1;2	1.00 ± 0.09	0.63 ± 0.07	3.42 ± 0.44	4.46 ± 1.07
SULT2;1	0.46 ± 0.04	0.55 ± 0.05	2.32 ± 0.31	2.88 ± 0.20
APR1	0.17 ± 0.01	0.69 ± 0.08	3.65 ± 0.41	4.10 ± 0.51
APR2	0.14 ± 0.00	0.36 ± 0.05	2.28 ± 0.21	3.89 ± 0.71
APR3	0.18 ± 0.01	0.59 ± 0.09	7.47 ± 0.92	13.98 ± 1.55
GGCT1;2	0.22 ± 0.02	1.12 ± 0.07	1.54 ± 0.09	0.98 ± 0.07
GST11	0.77 ± 0.10	0.40 ± 0.10	14.74 ± 3.16	5.22 ± 0.95
GRX480	1.00 ± 0.11	0.25 ± 0.03	4.15 ± 0.63	0.70 ± 0.11
Ethylene				
WRKY33	1.45 ± 0.11	0.64 ± 0.06	1.27 ± 0.05	1.58 ± 0.37
ACS6	0.95 ± 0.08	0.82 ± 0.13	1.22 ± 0.11	1.21 ± 0.14
ETR2	1.84 ± 0.21	2.07 ± 0.14	1.37 ± 0.08	1.66 ± 0.05
ERF1	0.92 ± 0.23	0.54 ± 0.10	1.57 ± 0.26	1.49 ± 0.23
Oxidative stress				
CSD1	1.21 ± 0.06	1.15 ± 0.13	4.61 ± 0.58	7.76 ± 1.11
CSD2	0.93 ± 0.05	1.02 ± 0.06	1.60 ± 0.19	2.58 ± 0.45
CCS	1.19 ± 0.06	1.23 ± 0.06	1.26 ± 0.04	1.79 ± 0.25
SOD2	0.68 ± 0.05	0.93 ± 0.04	1.42 ± 0.14	1.78 ± 0.24
CAT1	1.61 ± 0.05	1.34 ± 0.13	2.40 ± 0.11	1.54 ± 0.21
FSD1	0.69 ± 0.05	0.66 ± 0.08	1.30 ± 0.09	0.63 ± 0.24
PX	0.52 ± 0.12	0.72 ± 0.06	0.31 ± 0.02	0.03 ± 0.01
Hallmarks oxidative stress				
UPOX	2.04 ± 0.09	3.17 ± 0.34	8.85 ± 0.65	10.33 ± 2.15
DEFL	0.54 ± 0.06	0.76 ± 0.14	3.36 ± 0.46	1.29 ± 0.25
AT1G19020	0.59 ± 0.01	0.59 ± 0.05	2.40 ± 0.42	0.98 ± 0.10
AT1G05340	0.86 ± 0.05	1.02 ± 0.07	4.01 ± 1.01	4.53 ± 1.17
TIR	0.07 ± 0.01	0.2 ± 0.13	7.20 ± 1.28	0.80 ± 0.09

Table 6.3. Relative gene expression levels obtained in samples of shoots of *A. thaliana* at 10 days of culture in VAPs under different treatments. Values are mean normalized expression relative to the indicated treatment (set at 1.00) ± S.D. of at least four biological replicates, each containing at least one individual plant. Statistically significant at $p < 0.05$ treatment-induced changes in expression relative to the indicated treatment are marked by color (▮ = upregulation; ▮ = downregulation). For gene abbreviations see ***Supplementary Table 3***.

gene	WT — WT + *Pantoea*	WT — WT + As	WT + As — WT + *Pantoea* + As	cad2-1 — cad2-1 + *Pantoea*	cad2-1 — cad2-1 + As	cad2-1 + As — cad2-1 + *Pantoea* + As
As-related						
PHT1;1	1.26 ± 0.26	3.55 ± 0.48	1.54 ± 0.27	1.45 ± 0.40	1.54 ± 0.36	1.91 ± 0.55
NIP7;1	0.84 ± 0.05	0.52 ± 0.04	1.11 ± 0.11	1.46 ± 0.17	0.53 ± 0.11	0.86 ± 0.06
ACR2	1.29 ± 0.07	1.28 ± 0.08	1.12 ± 0.02	1.02 ± 0.05	1.25 ± 0.21	1.06 ± 0.08
AsI3	0.84 ± 0.06	1.46 ± 0.11	0.96 ± 0.03	1.00 ± 0.06	1.82 ± 0.16	1.48 ± 0.11
AsI4	1.00 ± 0.12	1.19 ± 0.09	1.13 ± 0.08	1.10 ± 0.10	2.73 ± 0.33	1.80 ± 0.17
Pi-related						
ACP5	0.39 ± 0.05	0.32 ± 0.04	2.41 ± 0.54	1.25 ± 0.13	14.34 ± 2.50	1.55 ± 0.18
MGD3	0.29 ± 0.04	0.22 ± 0.04	4.10 ± 0.87	1.03 ± 0.12	6.31 ± 1.29	2.80 ± 0.40
S-related						
GSH1	1.02 ± 0.07	1.02 ± 0.05	1.01 ± 0.05	0.99 ± 0.09	1.24 ± 0.15	0.75 ± 0.09
GSH2	1.22 ± 0.09	1.00 ± 0.06	1.14 ± 0.07	1.24 ± 0.11	1.17 ± 0.13	0.96 ± 0.05
PCS1	1.60 ± 0.35	1.05 ± 0.10	1.98 ± 0.14	0.89 ± 0.14	0.77 ± 0.04	1.06 ± 0.11
MT1a	0.97 ± 0.06	1.15 ± 0.05	1.19 ± 0.05	1.04 ± 0.06	2.13 ± 0.39	1.33 ± 0.10
MT2a	1.13 ± 0.04	0.94 ± 0.06	1.36 ± 0.10	1.00 ± 0.08	4.94 ± 0.45	1.46 ± 0.02
MT3	1.01 ± 0.01	1.03 ± 0.05	1.13 ± 0.08	0.93 ± 0.07	0.81 ± 0.08	1.00 ± 0.10
SULT1;2	1.16 ± 0.09	1.28 ± 0.11	2.19 ± 0.27	0.85 ± 0.09	5.08 ± 0.80	2.85 ± 0.56
SULT2;1	0.69 ± 0.05	0.73 ± 0.03	1.48 ± 0.20	0.83 ± 0.07	4.08 ± 0.61	1.91 ± 0.13
APR1	0.26 ± 0.02	0.22 ± 0.02	1.54 ± 0.02	1.08 ± 0.12	4.94 ± 0.56	1.81 ± 0.19
APR2	0.46 ± 0.07	0.31 ± 0.05	0.85 ± 0.12	1.14 ± 0.17	4.87 ± 0.54	1.44 ± 0.22
APR3	0.26 ± 0.03	0.22 ± 0.02	1.17 ± 0.08	0.86 ± 0.13	8.85 ± 1.33	2.19 ± 0.20
GGCT1;2	0.41 ± 0.02	0.31 ± 0.02	1.94 ± 0.33	0.81 ± 0.10	10.80 ± 2.85	2.32 ± 0.28
GST11	5.71 ± 4.64	1.27 ± 0.18	7.49 ± 1.04	2.97 ± 1.45	24.22 ± 6.35	2.65 ± 0.40
GRX480	1.48 ± 0.18	0.92 ± 0.03	7.78 ± 1.85	1.40 ± 0.15	14.16 ± 2.65	1.31 ± 0.16
Ethylene						
WRKY33	2.73 ± 0.53	1.17 ± 0.17	2.16 ± 0.16	1.20 ± 0.10	0.96 ± 0.07	2.70 ± 0.47
ACS6	0.99 ± 0.15	0.67 ± 0.04	1.53 ± 0.08	0.85 ± 0.08	0.85 ± 0.13	1.51 ± 0.14
ETR2	1.27 ± 0.16	2.05 ± 0.15	0.77 ± 0.07	1.43 ± 0.09	1.53 ± 0.11	0.93 ± 0.02
ERF1	3.15 ± 1.35	0.87 ± 0.09	3.16 ± 0.21	1.83 ± 0.35	1.48 ± 0.30	2.99 ± 0.38
Oxidative stress-related						
CSD1	1.24 ± 0.05	1.01 ± 0.04	1.13 ± 0.04	1.18 ± 0.13	3.87 ± 0.60	1.90 ± 0.22
CSD2	1.00 ± 0.07	0.76 ± 0.03	0.90 ± 0.04	1.09 ± 0.06	1.31 ± 0.19	1.45 ± 0.20
CCS	1.03 ± 0.06	1.42 ± 0.07	0.83 ± 0.05	1.07 ± 0.05	1.51 ± 0.06	1.18 ± 0.14
SOD2	0.65 ± 0.03	0.71 ± 0.03	0.97 ± 0.08	0.89 ± 0.04	1.49 ± 0.18	1.21 ± 0.13
CAT1	1.28 ± 0.08	1.44 ± 0.06	0.95 ± 0.05	1.07 ± 0.11	2.14 ± 0.12	0.61 ± 0.07
FSD1	0.92 ± 0.04	0.91 ± 0.06	0.72 ± 0.03	0.88 ± 0.11	1.72 ± 0.15	0.35 ± 0.11
PX	2.82 ± 1.52	1.52 ± 0.30	5.27 ± 1.40	3.89 ± 0.32	0.97 ± 0.06	0.75 ± 0.10
Hallmarks oxidative stress						
UPOX	0.76 ± 0.03	2.65 ± 0.33	1.06 ± 0.16	1.18 ± 0.12	11.51 ± 1.04	1.24 ± 0.21
DEFL	1.53 ± 0.27	1.36 ± 0.20	4.56 ± 0.48	2.16 ± 0.99	8.50 ± 1.43	1.97 ± 0.28
AT1G19020	1.64 ± 0.21	0.64 ± 0.05	4.13 ± 2.51	1.63 ± 0.14	2.59 ± 0.55	1.68 ± 0.14
AT1G05340	0.91 ± 0.06	1.49 ± 0.02	1.44 ± 0.16	1.07 ± 0.08	6.94 ± 2.13	1.63 ± 0.34
TIR	3.42 ± 1.47	0.20 ± 0.07	22.35 ± 5.73	10.15 ± 5.60	21.85 ± 3.88	2.50 ± 0.25

6.4 DISCUSSION

Stress constantly challenges organisms to adapt to a changing environment like As exposure, which provides a selective pressure for resistance genes. Metal resistance genes for example to As, copper and silver are ubiquitous in bacterial communities across different environments and occur in many different phyla (Pal et al., 2015). To our knowledge, *Pantoea* sp. IMH was the only bacterium found in the genus *Pantoea* with a high As resistance (Wu et al., 2013). Wang et al. (2016) demonstrated the function, involvement and temperature-dependence of different *ars* genes and operons in As V reduction and hence, in As tolerance of the IMH strain. In *Pantoea* sp. AV62 isolated from an As-polluted soil in the abandoned fabric of Nitrastur, (Langreo, 43°18'49"N, 5°42'05"W, Asturias, Spain) (https://www.ncbi.nlm.nih.gov/Traces/wgs/?val=NHBE01), our results confirmed As resistance that is related to one *arsC* gene (*arsC8*) and two *ars* systems with low sequence homology in *Pantoea* sp. AV62: a four gene *ars17* cluster (*arsR17B17C17H17*) and a three gene *ars19* cluster (*arsR19B19C19*) (**Fig 6.3**). At different time points, we also confirmed that both clusters are induced under As presence with *ars17* as major contributor at 30 °C and *ars19* at 15 °C, whereas the *arsC8* gene is suggested to play a minor role due to its low transcription levels. The two different genetic As resistance clusters allow *Pantoea* sp. AV62 to colonize changing ecosystems and render a flexible adaptation to resist As. The latter was evidenced in *A. thaliana* plants exposed to As in which inoculation caused an increase in the presence of As III in both roots and shoots (**Fig. 6.2**). In addition, in the shoots of the As-sensitive *cad2-1* mutants, a 4.5-fold increase in *Pantoea's arsC17* transcripts and a strong decrease in *arsC19* and *arsC8* transcripts confirms *arsC17's* contribution as predominant As V reductase at 22 °C (**Fig. 6.7**). As reported in literature, it is known that generally arsC's redox status is maintained by glutaredoxins (Grx) in gram-negative bacteria and by thioredoxins (Trx) in gram-positive bacteria (Messens and Silver, 2006). Our results confirm that arsC's recycling in the gram-negative *Pantoea* sp. AV62 probably relies on temperature-dependent *grx* expression: *grx1* appears to mediate for *arsC17* at 30 °C and both *grx2* and *grx3* for *arsC19* at 15 °C (**Fig. 6.5**). This shows a *grx* type-specific dependence for *arsC's* catalytic cycles and, therefore, in future complementation studies, both *arsC17* and *arsC19* should be cloned.

After reduction, As III can be extruded out of the bacterial cells or As III should be prevented from entering inside the cell, which is reflected in the differential regulation of transcripts for aquaporin (AapZ). As such, the As III present in the medium will be readily

available for plant uptake. To cope with As toxicity, *Pantoea* sp. AV62, as previously described for gram-negative bacteria (Messens and Silver, 2006), relies on the thiol metabolism for which sulfate uptake via CysZ is needed, correlating with the increased *cysZ* expression observed (**Fig. 6.5**). Sulfate is subsequently utilized for the synthesis of S-containing compounds in cells like GSH [*e.g.,* the cofactor for Grx (Zhang et al., 2014)]. Furthermore, inside our plant shoots, As exposure caused the bacterial AV62 strain to induce GSH biosynthesis (via increased *Gsh* expression) in both *Arabidopsis*' genotypes, supporting the hypothesis that *Pantoea* stimulates the GSH pathway upon As stress (**Fig. 6.7**).

An additional challenge related to As V in the biosphere is its similarity to the macronutrient Pi, resulting in competition between As V and Pi at the Pi transporter. Although Pi is undoubtedly one of the most biologically important nutrients, its availability in soils is rather limited (Holford, 1997). Therefore, plants have evolved mechanisms to maximize Pi accessibility, such as increased root hair growth, lateral root branching and the induction of Pi transporters and phosphatases (Pérez-Torres, 2008). In this respect, it has been proved that when Pi is limited, Pi transporters are induced and As V is incorporated preferentially into plant cells (Catarecha et al., 2007; Wu et al., 2011). However, organic phosphorus compounds in soil account for 30 to 50% of total soil phosphorus and are often considered natural alternatives to the use of mineral phosphate fertilizer (Dai et al., 2011). One of the major forms of organic soil phosphorous is phytate, a molecule of *myo*-inositol with six phosphate residues (George et al., 2009; Rugova et al., 2014). Phytases produced by soil bacteria have important functions in recycling soil phosphorus by releasing inorganic phosphates from organic phosphorus-containing molecules (Jorquera, 2008). In our case, it is interesting to observe how *Pantoea* sp. AV62's *agpP* phytase responded to As by increasing its expression at both temperatures after 12h of exposure. This is in accordance with the results of Suleimanova et al. (2015), who observed that metal ions positively regulate activity of *Pantoea* sp. 3.5.1 AgpP phytase. However, the decrease in *agpP* transcript levels at 24 h (**Fig. 6.6**) might indicate that, whereas all phytases increase bioavailability of essential metals like calcium, magnesium, zinc and manganese by breaking down metal ion-phytate complexes (Lei et al., 2013), too much metal ions can create a negative feedback loop of enzyme regulation. Therefore, phytase activity should be evaluated in the future and its potential as alternative to phosphate fertilizers in order to enhance agriculture or to increase As accumulation and tolerance in case of phytoremediation.

Investigating GSH's role in As resistance in more detail, we observed that the GSH-deficient *A. thaliana cad2-1* mutants differed in plant growth (**Fig. 6.9**), P and As accumulation (**Table 6.1**) and tolerance as compared to WT plants when exposed to As and/or inoculated with *Pantoea* sp. Under control conditions, the *cad2-1* mutant displayed typical characteristics due to its GSH deficiency. Concerning growth, only a decreased shoot fresh weight was detected in *cad2-1* mutants as compared to WT plants (**Fig. 6.9**). Gene expression data in shoots however, revealed *CAT1* induction in the mutant, possibly to compensate for the decrease in GSH's antioxidant capacity (**Table 6.2**). Due to its decreased GSH biosynthesis activities, the *cad2-1* mutant has been associated with accumulation of the GSH precursor cysteine (Parisy et al., 2007). This was reflected by the downregulated expression of S assimilation-related genes (*SULT2;1, APR1/2/3, GGCT1;2*) (**Table 6.2**). We also observed elevated P levels in *cad2-1* shoots (**Table 6.1**), explaining the downregulation of genes responsible for P_i release from membranes (*MGD3, ACP5*) (Abercromie et al. 2008). Finally, induction of the *ASI3* gene coding for a short-chain dehydrogenase/reductase (SDR) suggests the relevance of SDRs as scaffolds for an NAD(P)(H) redox sensor system (Kavanagh, 2008), crucial to control metabolic routes, transcription and signaling that are needed to compensate for the stress induced upon GSH deficiency.

Exposure to As for 10 days significantly inhibited growth in both genotypes but *cad2-1* mutants were more severely affected than WT plants, supporting increased As sensitivity in GSH-deficient plants (Liu et al., 2010). Since *cad2-1* shoots were already smaller in the control treatment than WT plants, it is clear that besides its role in defense against pollutants and stress mitigation, GSH is also involved in maintaining other metabolic processes important for normal plant growth. Both genotypes were able to reduce As V to As III but detoxification occurred more efficiently in WT plants as evidenced by As speciation and the general lack of gene inductions in WT plants, whereas almost all investigated genes were induced in shoots of As-exposed *cad2-1* mutants (**Table 6.3**). Despite the induction of the S metabolism and As sequestering strategies based on *MT1a* gene expression, As-induced oxidative stress in the GSH-deficient mutant was indicated by As V-inducible genes (*ASI3, AIS4*), enzymatic ROS scavenging (*CSD, FSD, CAT, AOX*) and the upregulation of all investigated oxidative stress hallmark genes (*UPOX, DELF, AT1G19020, AT1G05340, TIR*).

To unravel the potential of plant-associated bacteria in As tolerance and accumulation, it is crucial to find effective plant–bacterium interactions tailored for the specific purpose (e.g. phytoremediation, crop production, ripening …) (Remans et al., 2012). In this experimental

work, the potential of plant-associated bacteria to improve growth under As exposure was first estimated in **Chapter 2** by screening *in vitro* phenotypic characteristics in *Pantoea* sp. AV62 for its production of plant-growth-promoting (PGP) substances (e.g., IAA), stress-relieving properties (e.g. ACC-deaminase), increased nutrient uptake (e.g. nitrogen fixation, organic acids, P solubilization, siderophores) and sequestration and reduction of As. Preliminary results in **Chapter 4** and **Chapter 5** show a potential role for *Pantoea* sp. AV62 to increase pollutant concentrations in *S. atrocinerea* plant tissues. Inoculation of both *Arabidopsis* genotypes with *Pantoea* sp. AV62 bacteria favored seed germination in control and As conditions (data not shown) and increased the root lateral number (**Fig. 6.8**) as is generally observed for PGP rhizobacteria (Contesto et al., 2008). These bacteria can colonize the rhizosphere of *Arabidopsis* plants and stimulate plant growth by making nutrients more available to plants or by releasing phytohormones (Bloemberg and Lugtenberg, 2001; Weyens et al., 2009b).

Our results confirm *Pantoea's* PGP (**Fig. 6.9**) and As reduction (**Fig. 6.10**) capacities in both genotypes and increase P uptake in WT as was evidenced in WT shoots (**Table 6.1**). Protection by *Pantoea* against As toxicity was confirmed in WT plants with elevated As levels (**Table 6.1**) accompanied by mitigation of the fresh weight loss observed in non-inoculated As-exposed plants (**Fig. 6.9**). In response to these higher As levels, WT shoots showed elevated expression of oxidative stress hallmark genes upon inoculation, but the plants were able to efficiently cope with the As-induced stress by inducing ethylene-, PC-, S- and P_i-related genes (**Table 6.3**). In the As-sensitive *cad2-1* mutant on the contrary, inoculation with *Pantoea* resulted in decreased As translocation and lower As accumulation in shoots (**Table 6.1**). Furthermore, the fresh weight loss was equal to the non-inoculated mutants containing lower As concentrations (**Fig. 6.9**) despite the elevated ethylene-, PC-, S- and P_i-related gene expression (**Table 6.3**). This might also be a consequence of a differential As speciation, with higher As III percentage in inoculated plants as compared to non-inoculated plants (**Fig. 6.10**). Arsenic enters the protoplasm via the roots; whereas As V as P_i analogue utilizes the P_i route, As III goes through silicon transporters (Ali et al., 2009). The high-affinity PHOSPHATE TRANSPORTER1;1 (PHT1;1) has a major role in As V uptake in *Arabidopsis* (Shin et al., 2004). Interestingly, Catarecha et al. (2007) identified an As V-tolerant mutant *pht1;1-3* that harbors a semidominant allele of *PHT1;1*, resulting in a slow rate of As V uptake allowing the detoxification machinery to cope more efficiently with As and increase its accumulation in the plant. Interestingly, when As V uptake causes P_i deprivation, P_i starvation-induced genes such

as monogalactosyldiacylglycerol synthase encoded by *MGD3* and a type 5 acid phosphatase (*ACP5*) can stimulate the release of P_i from plasma membranes by hydrolyzing phospholipids, a major P_i pool in plant tissues (Abercrombie et al., 2008)*.* In *Arabidopsis*, *ACP*5 has also been shown to be induced by hydrogen peroxide (H_2O_2), and is thought to be involved in ROS scavenging (del Pozo et al., 1999). Our results show an upregulation of *PHT1;1* in WT plants upon As exposure (**Table 6.3**), which was even higher upon inoculation with *Pantoea* (**Table 6.3**), whereas the *cad2-1* mutant did not show significant changes in *PHT1;1* expression. This reflects that As toxicity is sensed in different ways in both genotypes. While *PHT1;1* induction causes the less As-sensitive WT plants to accumulate As, it is also able to maintain its P_i homeostasis and plant growth. Interestingly, to maintain its elevated basal P levels in inoculated WT shoots, WT plants invested in both strategies upon As exposure: elevated P_i uptake (*PHT1;1*) and the P_i starvation response (*MDG3, ACP5*). In the *cad2-1* mutant however, *PHT1;1* is not significantly induced in order to prevent high As accumulation into the cells. Consequently, P_i uptake and hence, plant growth, were compromised despite the mutant's induction of *MDG3* and *ACP5*. Inoculation with *Pantoea* sp. resulted in a more pronounced phosphatase response, but this did not protect the mutants against As toxicity; transcript levels of the oxidative stress hallmark genes were even higher (**Table 6.3**). A possible explanation to this can be related to the As speciation state in plant tissues.

Since As levels are two to three times higher in WT compared to *cad2-1* plants, this confirms that sufficient GSH (and hence PC) levels are available for As detoxification in WT plants (Kumar, 2008). This also suggests that a repressive effect of As V on P_i starvation responses may reflect a regulatory mechanism to protect plants from the extreme toxicity of As. Similar to Catarecha et al. (2007), our results suggest that under a certain As accumulation threshold, plants have evolved an As V sensing system, whereby As V and P_i signaling pathways oppose each other to protect the plant from As toxicity. Nevertheless, the signaling mechanisms by which plants distinguish between As V and P_i are unknown (Abercrombie et al., 2008).

In aerobic soils, As V is the predominant form (95%) but reduction processes taking place in the rizhosphere can increase As III presence (Hu et al., 2015). Once inside the plant, As III is the predominant form (Liu et al., 2010). Isayenkov and Maathuis (2008) identified the *A. thaliana* aquaglyceroporin NIP7;1 as a pathway for As III uptake. Our results showed that both *Arabidopsis* genotypes responded in a similar way to As III, limiting its entrance in the cell by repressing *NIP7;1* expression. This probably guarantees As III uptake to levels the plants are

able to detoxify, which is in accordance with the higher repression observed in the basal transcript levels of the As-sensitive *cad2-1* (**Table 6.2**). Alteration of As transport via As III transporters offers a possibility to genetically engineer plants, either to increase uptake for phytoremediation purposes or to reduce As III in the food chain (Isayenkov and Maathuis, 2008, Xu et al., 2015).

Once inside the cell, the majority of As V is reduced to As III by As reductase (ACR) (Bleeker et al., 2006; Dhankher et al., 2006); non-enzymatic reduction can also occur via GSH but the enzymatic rate of ACR is much higher (Duan et al., 2005). Reduction is an important step in As V detoxification through either As III efflux or complexation of As III with thiol compounds. The plant ACR2 enzyme uses GSH and glutaredoxin (GRX) as electron sources (Ellis etal., 2006; Duan et al., 2007), where the catalytic cycle involves the formation of a mixed disulfide between GSH and ACR2 by GRX (Mukhopadhyay et al., 2000). Although we did not observe changes in the transcript levels of *ACR2*, the decreased ability of *cad2-1* to reduce As V confirms the GSH-dependency of As V reduction (Bleeker et al., 2006; Dhankher et al., 2006; Ellis et al., 2006; Duan et al., 2007) and the fact that plants may also possess other enzymes with As V reductase activity (Sánchez-Bermejo et al., 2014). Furthermore, the fact that more As III was present in the tissues of inoculated as compared to non-inoculated plants (**Fig. 6.10**) highlights the bacterial role in As reduction and availability.

Arsenite conjugates with thiol-containing antioxidants like GSH and PCs (Sneller et al. 1999; Liu et al., 2010). This detoxification however, compromises the sulfate reduction pathway and hence induces gene expression to generate enough cysteine to cope with toxic As concentrations (Lafuente et al., 2015; Srivastava et al., 2015). Taking this into account, As detoxification indicates a critical importance for the S metabolism in determining plant survival in As-contaminated soils. Sulfate is the main form of S available to plants. The most important transporters involved in roots are the high-affinity SULTR1;1 and SULTR1;2 proteins. Sung et al. (2009) already reported upregulation of a sulfate transporter in As-exposed plants. Although it is not clear whether As III and As V equally affect the expression of these transporters, Chakrabarty et al. (2009) showed that at least *SULTR1;2* was induced by both forms of As. Next, sulfate is reduced in order to be used for the synthesis of cysteine in the primary sulfate assimilation pathway (Droux, 2004; Takahashi et al., 2011). This thiol-containing amino acid is subsequently incorporated into different compounds including GSH, PCs and MTs, important not only in metal chelation, but also in detoxification of xenobiotics and antioxidative defence (Anjum et al., 2014). The biosynthesis of GSH and PC that is typically

induced upon As exposure requires adequate supplies of the GSH-building blocks glutamate, cysteine and glycine. In *Arabidopsis*, cysteine is by far the limiting substrate for GSH biosynthesis (Finnegan and Chen, 2012) and requires the presence of a 5'-adenylylsulfate reductase (encoded by *APR*), which was induced in *Arabidopsis* in response to As V (Abercrombie et al., 2008). Concerning glutamate, the enzyme γ-glutamyl cyclotransferase (encoded by *GGCT2;1*) protects *Arabidopsis* plants from As toxicity by recycling glutamate to maintain GSH homeostasis (Paulose et al. 2013). The fact that these genes involved in S metabolism were not induced (*SULTR1;1/1;2*) or even downregulated (*APR1/2/3, GGCT2;1*) in the less As-sensitive WT plants suggest that this part of the S metabolism was sufficiently active upon As exposure. Nevertheless, inoculated WT plants did induce S-related gene expression upon As exposure. This coincides with the higher As-accumulation and induction of PC synthesis (*PCS1*) to ensure a more efficient As detoxification. Although no GSH or PCs were measured in this experiment, Liu et al. (2010) observed that in WT *A. thaliana* plants As was mainly (69%) complexed as As III-PC$_4$, As III-PC$_3$, and As III-(PC$_2$)$_2$. In the *cad2-1* mutant, where synthesis of GSH is hampered, a more general induction of the S assimilation pathway seems a response to the elevated As, which was even stronger upon inoculation (**Table 6.3**). Liu et al. (2010) also noted that *cad2-1* was more sensitive to As V than WT plants and As III-PCs only accounted for a small percentage (25%) of the total As in the plants. A possible explanation for the decreased As III proportions in mutant plants is the lack of As III complexation by GSH and PCs, which might cause reoxidation to As V (Schmöger et al., 2000). This lack of complexation in plant tissues is most likely the primary reason for the different As V sensitivity between both genotypes, because free As III is disruptive to the cell metabolism by binding critical thiols in proteins and hence generating oxidative stress (Hughes, 2002). Related to this last, the expression of As-inducible *AI3* and *AsI4*, which are proved to be responsive to As III and not to As V (Catarecha et al., 2007), were only upregulated in the *cad2-1* mutant. Interestingly, the *cad2-1* mutant induced *MT1a* and *MT2a* as alternatives to its limited capacity to scavenge As by GSH and PCs (**Table 6.3**). An important enzyme in the metabolism of GSH are glutathione-*S*-transferases (GSTs), a diverse family of enzymes that exhibit As-dependent expression (Norton et al., 2008; Chakrabarty et al., 2009; Huang et al., 2012b). One possible mechanism of action of GSTs in the presence of As is performing conjugation of the metalloid with GSH (Pandey et al., 2015) or mitigate oxidative stress caused by As (Schutzendubel and Polle, 2002). In our experimental setup, upregulation of *GST11* seems to be involved in tolerance to higher As concentrations in the WT plants. In the *cad2-1* mutants, under a deprived GSH scenario,

GST11 gene upregulation seems to correspond to cell protection from oxidative stress damage. Other important GSH-dependent redox enzymes are glutaredoxins (GRX) that transfer electrons from GSH to oxidized cysteine residues, thereby contributing to protein integrity and regulation (Fernandes and Holmgren, 2004). In this study we observed that *GRX480* is involved in the As response of both *Arabidopsis* genotypes, especially in *cad2-1* mutants.

It has been shown by Fu et al. (2014) that exposure to As causes the induction of genes encoding for ethylene-signaling components. The hormone ethylene participates in signaling cascades regulating both development and responses to stress under several biotic and abiotic stress conditions including metal stress (Schellingen et al., 2015; Keunen et al., 2016). In *A. thaliana*, it was suggested that a regulatory interaction between S assimilation and ethylene contributes to As tolerance by maintaining high GSH levels (Fu et al., 2014). However, ethylene's specific role in *Arabidopsis* exposed to As has not been reported before. In this study, genes coding for ethylene biosynthesis (*ACS6*) and ethylene-signaling components (*ETR2*, *ERF1*, *WRKY33*) were differentially regulated upon As exposure in non-inoculated and inoculated plants, but similarly between both genotypes. Both genotypes induced *ETR2*, whereas inoculation resulted in additional inductions of *ACS6, ERF1* and *WRKY33*. These data suggest that *Pantoea* sp. exerts a role in metabolizing the ethylene produced by the plant under As stress. Nevertheless, more research on the hormone levels and its precursor ACC are needed to clarify these data of *Arabidopsis-Pantoea* interaction under As exposure.

It is known that As stimulates the antioxidant capacity in plants, representing a secondary defense mechanism against oxidative stress that corresponds with a high As accumulation and a lack of toxicity symptoms in species like *Pteris vittata* (Cao et al., 2004). In general, our results did not show any upregulation of antioxidant genes in the WT plants upon exposure to As. However, they were activated in the As-sensitive mutant *cad2-1*, where these defense mechanisms may have been overwhelmed, leading to cellular damage by ROS and the observed reduction in growth as compared to the control condition.

6.5 CONCLUSIONS

Upon exposure to As, both, *Pantoea* sp. AV62 and *A. thaliana* showed molecular and physiological changes to cope with As toxicity. *Pantoea* sp. AV62 is equipped with two *ars* clusters that operate at different temperatures, which allows this bacterium to grow under changing environmental conditions. In *A. thaliana*, differential expression of particular genes

demonstrates that As stress significantly affects biological processes, but interestingly, we see that As V stress represses genes induced by Pi starvation *in planta*. Moreover, it is clear that GSH plays an essential role in the stress responses to As.

Plant-microorganism interactions are key to plant growth and development in the environment. Therefore, obtaining knowledge of how plants acquire tolerance to As and how their associated bacteria contribute to this process is essential for developing novel strategies for efficient phytoremediation. In this study, it was proved that inoculation of *A. thaliana* with bacteria isolated from *Salix atrocinerea* growing in As-polluted soil can also increase As accumulation and tolerance in this plant. Inoculation of *Pantoea* sp. to *A. thaliana* modifies root morphology, increases P accumulation, and alters the speciation pattern of As in plant tissues, which was translated into a differential accumulation of As and gene expression. Based on the experimental verification of these acclimation strategies in long-term exposure setups to As, more tolerant plant species could be achieved by molecular approaches or diminish their sensitivity by fostering plant-microbe interactions. Nonetheless, information provided by *in vitro* experiments will have to be validated in growth systems and species that are more relevant in economic terms (e.g. crops), where an integration of environmental effects on plants or bacteria and their interactions is needed to optimize this plant–bacteria system.

7 Supplementary tables

Supplementary Table 1. Primer sequences used for the real time RT-PCR analyses in *S. atrocinerea*. All primers have a product size between 90-150 bp and an efficiency between 80-120 % and $R^2 > 0.9$.

Gene	Gene description	*S. purpurea* ortholog locus or NCBI annotation	Primer sequence F/R (5′-3′)		
Reference genes					
OUT	OTU-like cysteine protease family protein	SapurV1A.0615s0200.1	GGCAGTGGTTCCTCTTCGAA ATCCCCATCTTTCGCAGTCG		
ACT7	Actin 7	SapurV1A.0231s0320.1	CTGTCCTTTCCCTGTATGCCA GTCACGACCAGCAAGATCCA		
α-TUB2	Alpha-tubulin 2	SapurV1A.0598s0030.1	CCAAGCGAGCATTTGTCCAC CCCTCGTCATCACCACCTTC		
DNAJ	Chaperone protein DnaJ 49	SapurV1A.0212s0110.1	GCTCCCGGTTCTTCTTATTTTCC AAATTAACCCCTCTCTGCGTAGT		
EF1α	Elongation factor 1-alpha	SapurV1A.0023s0300.1	ACCAGATTTCCGAGCCCAAG TTGGCCCAAAAGTGCAAACC		
ARF2	ADP-ribosylation factor 2	SapurV1A.0014s0160.1	TGGGGCTGTCTTTCACCAAG GGTCACAATCTCACCGAGCT		
Arsenate transport					
HAPO4	High-affinity phosphate transporter 4	HQ228362.1	GAACGACGAGCACCTGGTT ACGGGTTCTATTCGCCTTGA		
NA-DPHOT	Sodium-dependent phosphate transporter	SapurV1A.0139s0260.1	CAGCCACTTATCCCCAGCAA TCAAGGCGAATAGAACCCGT		
PHO1	Phosphate transporter PHO1-like protein	SapurV1A.0063s0550.1	AGAGGCTGCGATGTTGAACA GTCTGAAGCAAGGCGAGTCA		
Arsenite transport					
BORON	Boron transporter	SapurV1A.0014s0200.1	TCATTCGGGGAACAACTGGAG ACTGTCGGCTCTGCAACTC		
NIP1	Aquaporin NIP1.1	SapurV1A.0029s0170.1	CAAGGTTGTGACTCTTCCAGGA GACAGCAGGGTTGAAATGGG		
SIP1	Aquaporin SIP.1	SapurV1A.1058s0060.1	GCCAGTTCAGTACAAGCACATG TGCAGCAGAGGGTTTCGAG		
SILICON	Silicon 1	SapurV1A.1225s0080.1	GGTAGCAGTCTCAGCAGGTG TGAAAGGTTCCCAGCAACTGT		
Arsenate reductases					
CDC25-1	Tyrosine phosphatase	SapurV1A.0142s0310.1	ACGGCATCTTTAGGTCTGGTT TACGGCTCGGGACATAGACA	97	92.9%
CDC25-2	Tyrosine phosphatase	SapurV1A.0243s0430.1	TCAACTTTCACCACAGAAGACCT CACTAGTTGACGAGCCAGGA	147	89.9%
Thiol chelating response					
GR	Glutathione reductase	SapurV1A.0056s0770.1	ACGAAATGAGGGCTGTGGTT CCTCTCCATGATCTGTGCGA		
GS	Glutathione synthetase	SapurV1A.1124s0080.1	GCTGTCAAGTGCCCATCCAT CAGACTCCATAAGCCAGCGA		
PCS	Phytochelatin synthase	SapurV1A.0160s0210.1	GTGGAAGGGTATTGCTGTAAGGA TGAGATGAAGGAACCAGCACA		
GST	Glutathion S-transferase	SapurV1A.0016s1070.1	CGGTTCTTGGCTGGAGATGA CCTCCCCACATTTTCCCTGG		
MT1A	Metallothionein	*S. matsudana* EF157299.1	CTTCGGTGCTGAGAATGGCT CTGCTTTGTTGGGACCATGC		
ABCG	ABC transporter G	SapurV1A.0258s0220.1	AGGCTTGGATTCTACAACTGCT TGGCTGGTGGATTGTTGTCA		
CAX2-1	Vacuolar cation/proton exchanger 2	SapurV1A.1071s0020.1	TCTTGCAATCGTCGTCCACA ACCTAAACGCTCAGCCAAGG		
CAX2-2	Vacuolar cation/proton exchanger 2	SapurV1A.0338s0120.1	TTGTTGGTGCTTGGATGTGC GCAGGACAGCAGGAAAGAG		
WBABCT	White-brown-complex ABC transporter	SapurV1A.0084s0020.1	GCAAGAGGTGGTAGGACTGT ACACCCATCCGACAAAACCA		

Supplementary Table 1 **Continued**. Primer sequences used for the real time RT-PCR analyses in *S. atrocinerea*. All primers have a product size between 90-150 bp and an efficiency between 80-120 % and R^2 > 0.9.

Gene	Gene description	*S. purpurea* ortholog locus or NCBI annotation	Primer sequence F/R (5′-3′)
Flavonoid Synthesis			
CHS1	Chalcone synthase	SapurV1A.0820s0070.1	CATTCCGTGGCCCTAGTGAC CGGAGCCTACAATGAGAGCA
CHS2	Chalcone synthase 2	SapurV1A.0056s0660.1	AACTGCGAGCCACTAGACAC AAAAGCACACCCCACTCCAA
CHS3	Chalcone synthase 3	SapurV1A.0820s0080.1	GCGGCCCAGACTATTCTACC AGCCTCGGTCAGACTCTTCT
F3H	Flavanone 3-hydroxylase	SapurV1A.1567s0010.1	TCTTGTCGGAGGCTATGGGA TCGGTATGGCGTTTGAGTCC
FLH	Flavonoid 3'-hydroxylase	SapurV1A.0426s0030.1	TCGGCTTCTGTTGCTTCTCA TGCAAACACAAGGTCCTGGT
2HFLR	Dihydroflavonol 4-reductase	SapurV1A.0188s0360.1	GCCACCATTCACGATCTTGC ACTCGCCAAATCCTCATCGA
FLS	Flavonol synthase	SapurV1A.1087s0040.1	TCCCAACCCAGATTGTGTCG CAAATAGGCCCCACTGCGAA
ANR	Anthocyanidin reductase	SapurV1A.0028s0410.1	TTCCCAGCAGCGTAAACCTG GGGCTCTGCAAACATCCTCT
ANS	Anthocyanidin synthase	SapurV1A.0260s0310.1	TGTTATGCACCTTGTCAACCATG TCCTGAAGCCTGATCGTTCG
Stress related			
ACCS	1-aminocyclopropane-1-carboxylate synthase	SapurV1A.2160s0020.1	GCAGCACCAACTTTTGTCTCA GGGGTTGTTCGTAGGGTGAA
AIP-1	Arsenite-inducible RNA-associated protein AIP-1-related	SapurV1A.0229s0030.1	CTTGCCAGTTGAAGGTGTGC ACAATCTTTTCCGTTCCTCAAGG
ER	Ethylene receptor	SapurV1A.0052s0240.1	TACCATACACCTGCCCACTG GTAGTAGAGGTACACGAACAGCA
CSA	Cellulose synthase A catalytic subunit 9	SapurV1A.0828s0050.1	TCACAGTCACATCCAAGGCA TCCAGCAACAACTCCAACGA

Supplementary Table 2. Primer sequences used for the real time RT-PCR analyses in *Pantoea* sp. All primers have a product size between 90-150 bp and an efficiency between 80-120 % and $R^2 > 0.9$.

Annotation	Gene description	Forward (5'-3')	Reverse (5'-3')
Reference gene			
16S	16S ribosomal RNA	TGCATTTGAAACTGGCAGGC	AGCGTCAGTCTTTGTCCAGG
As tolerance			
arsH17	Arsenic resistance protein ArsH	AGTGCTCTGGTCTGAAGGGA	CGTCAGCGGTATCCAGTCA
arsC17	Arsenate reductase	TGGTAAACGCTCCCTTCTGC	AATCGTCCGGTGGTGGTAAC
arsB17	Arsenic efflux pump protein	GCACGGTAGGCATGTTGTTC	GATTACCTTTCTGCCGCCCT
arsR17	Arsenical resistance operon repressor	GCCTGTCGATAAGCAGTCCA	AAAAGGGGAGCTTTGCGTCT
arsC19	Arsenate	GCTTCTCCGTCCTCTTTGCT	CTGCTGATTAACCGCCCAATC
arsB19	Arsenic efflux pump protein	ATCTGCACCACACCACTCAG	CTGGTGCTGGTTATCTGGCA
arsR8	Arsenical resistance operon repressor	ACCCCGCTTCAACTGTTCAA	ACGGGATATTTTCGGCTGGG
arsC8	Arsenate reductase	TCACTCTTCGTTTCCTCCGC	CGAGGTGGTGCTCTATCTGC
As transporters			
aqpZ	Aquaporin Z	ACCAACACCTCGGTCAATCC	TCAGCCAGAACATCCACAGC
pit1	Low-affinity inorganic phosphate transporter	GTTTCGACCACCCACATCCT	ACAATACATACCGGCAGCGT
pit2	Low-affinity inorganic phosphate transporter	TGTTGGCAGCAGTCAGTTCA	ATCTGGTTACGGGTGTTGGC
S-related			
cysZ	Sulfate transporter, CysZ-type	GCATGATGGCGAGGTTCAGA	GCGACTACCCGTTTGATAACC
cys1	Cysteine synthase 1	AATGTTGTTGTTACCGCGCC	CCCTGCCAGCTTTATGTCGA
cys2	Cysteine synthase 2	GCGATCAGGGAATGGAAGGT	CTGATAATGGCCCAGCGGAT
cys3	Cysteine synthase 3	AGATTGTCGCCAGTAACCCG	CCGACGCCAGCAATAAACAC
gcl	Glutamate--cysteine ligase	GGAAAGCGGGAAAGAGACCA	AGGAAGCTGGAACTGATGGC
gr	Glutathione reductase	ATGAAGCTGGTCTGTGTCGG	ATCGCCACGGTATTGTCGAA
gsh	Glutathione synthetase	ACCCGCAGCAAAGAGAAACT	TGATGACGCCAAAGTTCGGA
grx1	Glutaredoxin 1	GTAAGCCAGTCACCACCGT	GCCCAGGTTTTCTTTGGTCC
grx2	Glutaredoxin 2	AGCTGGTGGTGATGCTCAAT	CGATATCCATGCTCTCCGGC
grx3	Glutaredoxin 3	CAAATAAGTCGTCGCAGCCG	GGACATGGCGAAACGTGAAG
trxR	Thioredoxin reductase	ACGGAAACTGTCACGACGAT	CGACGGCTTCTTCTATCGCA
trx1	Thioredoxin	CCGTGCCTGGGTTATCATCA	CCTTGCAAAATGATCGCCCC
trx2	Thioredoxin 2	CGCAGCATCCCGACCATTAT	TACAGTGACTCGTCCAGCCA
Phytase			
agpP	Glucose-1-phosphatase	CGACTAACTGGCTACGGATGT	CAGATCAAAACGCCTGAGC

Supplementary Table 3. Primer sequences used for the real time RT-PCR analyses in *A. thaliana*. All primers have a product size between 90-150 bp and an efficiency between 80-120 % and $R^2 > 0.9$.

Locus	Gene description	Annotation	Forward primer (5' – 3')	Reverse primer (5' – 3')
Reference genes				
AT2G28390	SAND family	SAND	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC
AT3G18780	Actin2	ACT2	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT
AT4G05320	Polyubiquitin 10	UBQ10	GGCCTTGTATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAAACATAGT
AT5G15710	F-box protein	FBOX	TTTCGGCTGAGAGGTTCGAGT	GATTCCAAGACGTAAAGCAGATCAA
AT5G60390	Elongation factor 1α	EF1A	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA
AT4G34270	Elongation factor 1α	TIP41-like	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA
AT5G25760	Ubiquitin conjugating enzyme21	UBC21	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC
As-related				
AT5G43350	Pi transporter	PHT1;1	GGTGACAAACTCGGACGGAA	CCCAACCAAAACCTGAAGAAGC
AT3G06100	Aquaporin	NIP1;7	GAGCATCGTCGTGTTTCTCG	CTCCTGAAATCGGTCCGGTAA
AT5G03455	Cycle regulator CDC25	ACR2	AGCTGGATCGCTACACTATGC	CAAGTAGGGCCACGAACCTG
AT4G13180	NAD(P)-binding Rossmann-fold	AsI3	AAGCAGATGTGTCAGACCCG	CACCTGCACAATTCACGACG
AT4G33540	Metallo-beta-lactamase	AsI4	CTATCTTGTCCGACGGGCTC	AAACGCCAGGAAGTGTGTCT
Pi-related				
AT3G17790	Acid phoshatase	ACP5	TTCGTGGTGTCTACGGGAGA	TCCCAAAACACTGTACCACTGT
AT2G11810	Monogalactosyl diacylglycerol synthase 3	MGD3	ATTCCGGTCAAGGTTCGAGG	GAGGATAATTGGGAGGCCGC
S-related				
AT4G23100	Glutamate-cysteine ligase	GSH1	CCCTGGTGAACTGCCTTCA	CATCAGCACCTCTCATCTCCA
AT5G27380	Glutathione synthetase	GSH2	GGACTCGTCGTTGGTGACAA	TCTGGGAATGCAGTTGGTAGC
AT5G44070	Phytochelatin synthase	PCS1	TGCCAAGGAGCTGAAATCTT	ACCGTGCCTTCAGAGTCATC
AT1G07600		MT1a	AACTGTGGATGTGGCTCCTC	CAGTTACAGTTTGACCCACAGC
AT3G09390	Metallothionein	MT2a	ACCCTGACTTGGGATTCTCC	GCGTTGTTACTCTCCCCTGA
AT3G15353		MT3	TCGACATCGTCGAGACTCAG	CACTTGCAATTTGCGTTGTT
AT1G78000		SULT1;2	AGGCTTCCTCGGGATCAAGA	TAGTCTGCCAATTCCAGCCG
AT5G10180	Sulfate transporter	SULT2;1	CTTCCGTTGAGATCGGACTTCTT	CCAAGCGTTTCTATCCCTGGAC
AT4G04610		APR1	TCTTGTGACTTTGAGCAGGCAG	CCACGGAGCATAAAGCACGA
AT1G62180	Disulfide isomerase-like	APR2	TCGAGTTTCTCTCGTTCTGGAGC	TCTGAGACAAATGGGTTCGATCA
AT4G21990		APR3	GCTTCCCTTCTTCAGATCTCAAAGT	GACAAACTCAGAGAAGCCGCAG
AT5G26220	γ-glutamyl cyclotransferase	GGCT1;2	CGTGTCTTCGATCTAGCGTGT	ACACAATAAGCAGCACCCCA
AT1G02920	glutathione s-transferase	GST11	AGTTTTCGGTCACCCAGCTT	CAAAAGGGTTGCGGAAGATGA
AT1G28480	Glutaredoxin	GRX480	GGGAAACGGCGAGAGAGTTC	CCTCACCACATGACACATGCA
Ethylene				
AT2G38470	Transcription Factor	WRKY33	TCATCGATTGTCAGCAGAGACG	CCATTCCCACCATTTGTTTCAT
AT4G11280	ACC Synthase	ACS6	TTAGCTAATCCCGGCGATGG	ACAAGATTCACTCCGGTTCTCCA
AT3G23150	Ethylene Response	ETR2	TTCGAACCGGGCAGTTACAC	AATGGCGGTAAGGCAATCG
AT3G23240	Ethylene Response Factor	ERF1	TCCTCGGCGATTCTCAATTTT	CAACCGGAGAACAACCATCCT

Supplementary Table 3 Continued. Primer sequences used for the real time RT-PCR analyses in *A. thaliana*. All primers have a product size between 90-150 bp and an efficiency between 80-120 % and R^2 > 0.9.

Locus	Gene description	Annotation	Forward primer (5' – 3')	Reverse primer (5' – 3')
Oxidative stress-related				
AT1G08830	Cytosolic copper/zinc superoxide dismutase	CSD1	TCCATGCAGACCCTGATGAC	CCTGGAGACCAATGATGCC
AT2G28190	Chloroplastic copper/zinc superoxide	CSD2	GAGCCTTTGTGGTTCACGAG	CACACCACATGCCAATCTCC
AT1G12520	Copper-zinc superoxide dismutase	CCS	TTCACAGCATTAACACAACCCTCA	CAAGCCTTGTCGGTGGTTGA
At4G27940	CSD2Manganese tracking factor	SOD2	ACGCCTTTGGAGAGGGACTAAT	CCGGTTGCGGAACATATCAT
AT1G20630	Catalase	CAT1	AAGTGCTTCATCGGGAAGGA	CTTCAACAAAACGCTTCACGA
AT4G25100	Fe-superoxide dismutase	FSD1	CTCCCAATGCTGTGAATCCC	TGGTCTTCGGTTCTGGAAGTC
AT5G64100	Peroxidase	PX	GACTCTTGTTGGCGGACACA	AATCAGCGGCACAAAACTCG
Oxidative stress hallmark proteins				
AT2G21640	Unknown	UPOX	GACTTGTTTCAAAAACACCATGGAC	CACTTCCTTAGCCTCAATTTGCTTC
AT2G43510	Defensin-like	DEFL	ATGGCAAAGGCTATCGTTTCC	CGTTACCTTGCGCTTCTATCTCC
AT1G19020	Unknown	Unknown	GAAAATGGGACAAGGGTTAGACAAA	CCCAACGAAAACCAATAGCAGA
AT1G05340	Unknown	Unknown	TCGGTAGCTCAGGGTAAAGTGG	CCAGGGCACAACAGCAACA
AT1G57630	Toll-Interleukin-Resistance	TIR	ACTCAAACAGGCGATCAAAGGA	CACCAATTCGTCAAGACAACACC

CONCLUSIONS

Effect of Salix atrocinerea root-associated bacteria on the phytoremediation of arsenic-polluted soils

8 Conclusions

In addition to the specific conclusions within each chapter, a summary of the major contributions of this PhD thesis is presented and organized according to the main objectives defined in the introduction (**Chapter 1**).

1. The interactions between roots, microbes and soils have an effect on the root microbiome and explains the differences in the composition and diversity of the bacterial communities in the soil, rizosphere and endosphere of *S. atrocinerea*.

2. The presence of diverse plant-growth-promoting traits (e.g. production of hormones, siderophores...) in the root-associated bacteria of *S. atrocinerea*, indicates that the relationships at the cellular level between the bacteria and their host are implied in the accumulation and tolerance to pollutants by the plant. This opens new possibilities to assist plants via bioaugmentation under chronic pollutant-stress.

3. Under the presence of arsenate in hydroponic conditions, willow plants show a transcriptional regulation of genes involved in stress responses, arsenate reduction, nutrient transporters, glutathione synthesis and sequestration of As into the vacuoles, which coincides with a rapid arsenite presence and accumulation in root tissues, altered nutrient profile and *de novo* synthesis and increase of non-protein thiolic compounds which contribute to the plant tolerance to the metalloid.

4. Under *in vitro* conditions, it was shown how arsenic uptake by the plant relies on the As concentration available in the culture medium and, more importantly, on its speciation state; and this is affected by bacterial inoculation. Therefore, plant inoculation with *Pantoea* sp. or *R. erythropolis* constitutes a synergistic mechanism that alters arsenic toxicity by changing its speciation, mobility and accumulation in *S. atrocinerea*.

5. In pot, the reduced TE accumulation obtained in inoculated plants with *Pantoea* sp. or *R. erythropolis* in a polluted soil led to a higher biomass in comparison to non-inoculated plants, that in the long-term can increase the phytoextraction of TEs.

6. In field, bacterial inoculation resulted into a higher As and Pb accumulation in the plant and also a greater phytoextraction, together with a markedly higher Zn translocation. While in plants inoculated with *Pantoea* sp. this was achieved by a higher accumulation of TEs with a biomass decrease, in *R. erythropolis* it was obtained by a lower accumulation but higher biomass than in non-inoculated plants.

7. Given the Zn and Cd accumulation and root-to-shoot translocation rates in willow, together with the As and Pb accumulation in roots and its ability to grow in this area, *S. atrocinerea* is a suitable species to be used in the phytoremediation of soils polluted with these TEs. In addition, bioaugmentation with *Pantoea* sp. or *R. erythropolis* can reduce the time necessary for the soil remediation.

8. Considering that the results obtained in the lab cannot always be extrapolated to the field, the need of performing field assays to obtain successful results in phytoremediation is deduced. However, considerable knowledge on As tolerance, bioaugmentation-based remediation processes and plant-bacteria interaction has been gathered from controlled studies.

9. The selected bacteria have shown a great potential in phytoremediation, not only because of their ability to transform and detoxify pollutants like As, but also for their potential to synthesize enzymes like phytases or arsenate reductases as shown in their bacterial genome. Furthermore, bacteria were able to induce accumulation of certain molecules of interest in plants like tannins or hormones, which emphasizes their biocatalyst potential.

10. The use of bacteria isolated from *S. atrocinerea* growing in As-polluted soil can also increase As accumulation and tolerance in other plant species, as it is proved in *Arabidopsis thaliana*. Besides, cross-species bacteria inoculation provides more information about the mechanisms implied in bacteria-plant interaction that are needed to tailor specific pollutant-bacteria-plant remediation strategies.

REFERENCES

9 References

Abbas, G., Murtaza, B., Bibi, I., Shahid, M., Niazi, N.K., Khan, M.I., Amjad, M., Hussain, M., Natasha (2018) Arsenic Uptake, Toxicity, Detoxification, and Speciation in Plants: Physiological, Biochemical, and Molecular Aspects. International Journal of Environmental Research and Public Health 15, 59.

Abedin, M.J., Feldmann, J., Meharg, A.A. (2002) Uptake Kinetics of Arsenic Species in Rice Plants. Plant Physiol 128, 1120-1128.

Abercrombie, J.M., Halfhill, M.D., Ranjan, P., Rao, M.R., Saxton, A.M., Yuan, J.S., Stewart, C.N., Jr. (2008) Transcriptional responses of Arabidopsis thaliana plants to As (V) stress. BMC Plant Biol 8, 87.

Adriano, D.C. (2001) Trace Elements in Terrestrial Environments: Biogeochemistry, Bioavailability and Risks of Metals. 2nd Edition, Springer, New York, pp. 867.

Ahmad, P., M. Sarwat, N. A. Bhat, M. R. Wani, A. G. Kazi and L. S. Tran (2015). Alleviation of cadmium toxicity in Brassica juncea L. (Czern. & Coss.) by calcium application involves various physiological and biochemical strategies. PLoS One 10: e0114571.

Ahsan, N., Lee, D.-G., Alam, I., Kim, P.J., Lee, J.J., Ahn, Y.-O., Kwak, S.-S., Lee, I.-J., Bahk, J.D., Kang, K.Y., Renaut, J., Komatsu, S., Lee, B.-H. (2008) Comparative proteomic study of arsenic-induced differentially expressed proteins in rice roots reveals glutathione plays a central role during As stress. PROTEOMICS 8, 3561-3576.

Ahsan, N., Lee, D.G., Alam, I., Kim, P.J., Lee, J.J., Ahn, Y.-O., Kwak, S.S., Lee, I.J., Bahk, J.D., Kang, K.Y., Renaut, J., Komatsu, S., Lee, B.H. (2008) Comparative proteomic study of arsenic-induced differentially expressed proteins in rice roots reveals glutathione plays a central role during As stress. Proteomics. 8, 3561-3576.

Akhter, F., McGarvey, B., Macfie, S.M. (2012) Reduced translocation of cadmium from roots is associated with increased production of phytochelatins and their precursors. J Plant Physiol 169, 1821-1829.

Alcántara-Martínez, N., Figueroa-Martínez, F., Rivera-Cabrera, F., Gutiérrez-Sánchez, G., Volke-Sepúlveda, T. (2018) An endophytic strain of Methylobacterium sp. increases arsenate tolerance in Acacia farnesiana (L.) Willd: A proteomic approach. Science of The Total Environment 625, 762-774.

Ali, H., Khan, E., Sajad, M.A. (2013) Phytoremediation of heavy metals—Concepts and applications. Chemosphere 91, 869-881.

Ali, W., Isayenkov, S.V., Zhao, F.J., Maathuis, F.J. (2009) Arsenite transport in plants. Cell Mol Life Sci 66, 2329-2339.

Ali, W., Isner, J.C., Isayenkov, S.V., Liu, W., Zhao, F.J., Maathuis, F.J. (2012) Heterologous expression of the yeast arsenite efflux system ACR3 improves Arabidopsis thaliana tolerance to arsenic stress. New Phytol 194, 716-723.

Álvarez-Ayuso, E., García-Sánchez, A., Querol, X., Moyano, A. (2008) Trace element mobility in soils seven years after the Aznalcóllar mine spill. Chemosphere 73, 1240-1246.

Angiuoli, S.V., Gussman, A., Klimke, W., Cochrane, G., Field, D., Garrity, G., Kodira, C.D., Kyrpides, N., Madupu, R., Markowitz, V., Tatusova, T., Thomson, N., White, O. (2008) Toward an online repository of Standard Operating Procedures (SOPs) for (meta)genomic annotation. Omics 12, 137-141.

Anjum, N. A., M. Hasanuzzaman, M. A. Hossain, P. Thangavel, A. Roychoudhury, S. S. Gill, M. A. M. Rodrigo, V. Adam, M. Fujita, R. Kizek, A. C. Duarte, E. Pereira and I. Ahmad (2015). "Jacks of metal/metalloid chelation trade in plants—an overview." Frontiers in Plant Science 6(192)

Anjum, N.A., Aref, I.M., Duarte, A.C., Pereira, E., Ahmad, I., Iqbal, M. (2014) Glutathione and proline can coordinately make plants withstand the joint attack of metal(loid) and salinity stresses. Front Plant Sci 5, 662-662.

Anjum, N.A., Hasanuzzaman, M., Hossain, M.A., Thangavel, P., Roychoudhury, A., Gill, S.S., Rodrigo, M.A.M., Adam, V., Fujita, M., Kizek, R., Duarte, A.C., Pereira, E., Ahmad, I. (2015) Jacks of metal/metalloid chelation trade in plants—an overview. Front Plant Sci 6.

Antoniadis, V., Levizou, E., Shaheen, S.M., Ok, Y.S., Sebastian, A., Baum, C., Prasad, M.N.V., Wenzel, W.W., Rinklebe, J. (2017) Trace elements in the soil-plant interface: Phytoavailability, translocation, and phytoremediation–A review. Earth-Science Reviews 171, 621-645.

Appelhagen, I., Wulff-Vester, A.K., Wendell, M., Hvoslef-Eide, A.-K., Russell, J., Oertel, A., Martens, S., Mock, H.-P., Martin, C., Matros, A. (2018) Colour bio-factories: Towards scale-up production of anthocyanins in plant cell cultures. Metabolic Engineering 48, 218-232.

Argus, G.W. (1995) Salicaceae Willow Family: Part Two: Salix L. Willow. Journal of the Arizona-Nevada Academy of Science 29, 39-62.

Arnetoli, M., Vooijs, R., ten Bookum, W., Galardi, F., Gonnelli, C., Gabbrielli, R., Schat, H., Verkleij, J.A. (2008) Arsenate tolerance in Silene paradoxa does not rely on phytochelatin-dependent sequestration. Environmental Pollution 152, 585-591.

Asgher, M., Per, T.S., Anjum, S., Khan, M.I.R., Masood, A., Verma, S., Khan, N.A., (2017) Contribution of Glutathione in Heavy Metal Stress Tolerance in Plants, in: Khan, M.I.R., Khan, N.A. (Eds.), Reactive Oxygen Species and Antioxidant Systems in Plants: Role and Regulation under Abiotic Stress. Springer Singapore, Singapore, pp. 297-313.

Ashraf, M.A., Hussain, I., Rasheed, R., Iqbal, M., Riaz, M., Arif, M.S. (2017) Advances in microbe-assisted reclamation of heavy metal contaminated soils over the last decade: A review. J Environ Manage 198, 132-143.

Ayangbenro, A.S., Babalola, O.O. (2017) A New Strategy for Heavy Metal Polluted Environments: A Review of Microbial Biosorbents. International Journal of Environmental Research and Public Health 14, 94.

Aziz, M., Nadipalli, R.K., Xie, X., Sun, Y., Surowiec, K., Zhang, J.-L., Paré, P.W. (2016) Augmenting Sulfur Metabolism and Herbivore Defense in Arabidopsis by Bacterial Volatile Signaling. Front Plant Sci 7, 458-458.

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., Zagnitko, O. (2008) The RAST Server: Rapid Annotations using Subsystems Technology. BMC Genomics 9, 75.

Azizur Rahman, M., Hasegawa, H., Mahfuzur Rahman, M., Nazrul Islam, M., Majid Miah, M.A., Tasmen, A. (2007) Effect of arsenic on photosynthesis, growth and yield of five widely cultivated rice (Oryza sativa L.) varieties in Bangladesh. Chemosphere 67, 1072-1079.

Baath, E. (1998) Growth Rates of Bacterial Communities in Soils at Varying pH: A Comparison of the Thymidine and Leucine Incorporation Techniques. Microb Ecol 36, 316-327.

Badri, D.V., Chaparro, J.M., Zhang, R., Shen, Q., Vivanco, J.M. (2013) Application of natural blends of phytochemicals derived from the root exudates of Arabidopsis to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. J Biol Chem 288, 4502-4512.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., Vivanco, J.M. (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57, 233-266.

Bakker, P.A., Berendsen, R.L., Doornbos, R.F., Wintermans, P.C., Pieterse, C.M. (2013) The rhizosphere revisited: root microbiomics. Front Plant Sci 4, 165.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A. (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. Journal of computational biology : a journal of computational molecular cell biology 19, 455-477.

Barnawal, D., Bharti, N., Maji, D., Chanotiya, C.S., Kalra, A. (2012) 1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase-containing rhizobacteria protect Ocimum sanctum plants during waterlogging stress via reduced ethylene generation. Plant Physiology and Biochemistry 58, 227-235.

Bates, L.S., Waldren, R.P., Teare, I.D. (1973) Rapid determination of free proline for water-stress studies. Plant and Soil 39, 205-207.

Batista, B.L., Nigar, M., Mestrot, A., Rocha, B.A., Barbosa Junior, F., Price, A.H., Raab, A., Feldmann, J. (2014) Identification and quantification of phytochelatins in roots of rice to long-term exposure: evidence of individual role on arsenic accumulation and translocation. J Exp Bot 65, 1467-1479.

Beckers, B., Op De Beeck, M., Thijs, S., Truyens, S., Weyens, N., Boerjan, W., Vangronsveld, J. (2016) Performance of 16s rDNA Primer Pairs in the Study of Rhizosphere and Endosphere Bacterial Microbiomes in Metabarcoding Studies. Frontiers in Microbiology 7, 650-650.

Beckers, B., Op De Beeck, M., Weyens, N., Van Acker, R., Van Montagu, M., Boerjan, W., Vangronsveld, J. (2016) Lignin engineering in field-grown poplar trees affects the endosphere bacterial microbiome. Proceedings of the National Academy of Sciences, 201523264.

Ben Fekih, I., Zhang, C., Li, Y.P., Zhao, Y., Alwathnani, H.A., Saquib, Q., Rensing, C., Cervantes, C. (2018) Distribution of Arsenic Resistance Genes in Prokaryotes. Frontiers in Microbiology 9, 2473-2473.

Berg, G., Grube, M., Schloter, M., Smalla, K. (2014) Unraveling the plant microbiome: looking back and future perspectives. Front Microbiol 5, 148.

Berg, G., Krechel, A., Ditz, M., Sikora, R.A., Ulrich, A., Hallmann, J. (2005) Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. FEMS Microbiol Ecol 51, 215-229.

Bhaduri, A.M., Fulekar, M.H. (2012) Antioxidant enzyme responses of plants to heavy metal stress. Reviews in Environmental Science and Bio/Technology 11, 55-69.

Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., Kalayci, O. (2012) Oxidative Stress and Antioxidant Defense. The World Allergy Organization journal 5, 9-19.

Bleeker, P.M., Hakvoort, H.W., Bliek, M., Souer, E., Schat, H. (2006) Enhanced arsenate reduction by a CDC25-like tyrosine phosphatase explains increased phytochelatin accumulation in arsenate-tolerant Holcus lanatus. Plant J 45, 917-929.

Blindauer, C. A. and O. I. Leszczyszyn (2010). "Metallothioneins: unparalleled diversity in structures and functions for metal ion homeostasis and more." Nat Prod Rep 27(5): 720-741.

Blindauer, C.A. (2011) Bacterial metallothioneins: past, present, and questions for the future. JBIC Journal of Biological Inorganic Chemistry 16, 1011.

Bloemberg, G.V., Lugtenberg, B.J. (2001) Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr Opin Plant Biol 4, 343-350.

Bluemlein, K., Raab, A., Feldmann, J. (2009) Stability of arsenic peptides in plant extracts: off-line versus on-line parallel elemental and molecular mass spectrometric detection for liquid chromatographic separation. Anal Bioanal Chem 393, 357-366.

Bohari, Y., Lobos, G., Pinochet, H., Pannier, F., Astruc, A., Potin-Gautier, M. (2002) Speciation of arsenic in plants by HPLC-HG-AFS: extraction optimisation on CRM materials

and application to cultivated samples. Journal of Environmental Monitoring 4, 596-602.

Bolan, N., Kunhikrishnan, A., Thangarajan, R., Kumpiene, J., Park, J., Makino, T., Kirkham, M.B., Scheckel, K. (2014) Remediation of heavy metal(loid)s contaminated soils – To mobilize or to immobilize? Journal of Hazardous Materials 266, 141-166.

Bolaños, L., Lukaszewski, K., Bonilla, I., Blevins, D. (2004) Why boron? Plant Physiology and Biochemistry 42, 907-912.

Bonet, A., Lelu-Walter, M.A., Faugeron, C., Gloaguen, V., Saladin, G. (2016) Physiological responses of the hybrid larch (Larix x eurolepis Henry) to cadmium exposure and distribution of cadmium in plantlets. Environ Sci Pollut Res Int 23, 8617-8626.

BOPA. Boletin Oficial del Principado de Asturias. Generic reference levels for heavy metals in soils from Principality Of Asturias, Spain 2014 https://sede.asturias.es/bopa/2014/04/21/201406617.pdf.

Bowen, H.J.M. (1979) Environmental chemistry of the elements. London; New York Academic Press.

Bradford, M.A., Davies, C.A., Frey, S.D., Maddox, T.R., Melillo, J.M., Mohan, J.E., Reynolds, J.F., Treseder, K.K., Wallenstein, M.D. (2008) Thermal adaptation of soil microbial respiration to elevated temperature. Ecol Lett 11, 1316-1327.

Brettin, T., Davis, J.J., Disz, T., Edwards, R.A., Gerdes, S., Olsen, G.J., Olson, R., Overbeek, R., Parrello, B., Pusch, G.D., Shukla, M., Thomason, J.A., 3rd, Stevens, R., Vonstein, V., Wattam, A.R., Xia, F. (2015) RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. Sci Rep 5, 8365.

Briat, J.-F. (2010) Arsenic tolerance in plants: "Pas de deux" between phytochelatin synthesis and ABCC vacuolar transporters. Proceedings of the National Academy of Sciences 107, 20853-20854.

Bridges, C.C., Zalups, R.K. (2005) Molecular and ionic mimicry and the transport of toxic metals. Toxicology and applied pharmacology 204, 274-308.

Bücker-Neto, L., Paiva, A.L.S., Machado, R.D., Arenhart, R.A., Margis-Pinheiro, M. (2017) Interactions between plant hormones and heavy metals responses. Genetics and molecular biology 40, 373-386.

Buée, M., De Boer, W., Martin, F., van Overbeek, L., Jurkevitch, E. (2009) The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. Plant and Soil 321, 189-212.

Bulgarelli, D., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., Assenza, F., Rauf, P., Huettel, B., Reinhardt, R., Schmelzer, E., Peplies, J., Gloeckner, F.O., Amann, R., Eickhorst, T., Schulze-Lefert, P. (2012) Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. Nature 488, 91.

Burger, J., Gochfeld, M. (2016) Health Risks to Ecological Workers on Contaminated Sites - the Department of Energy as a Case Study. Journal of community medicine & health education 6, 427.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55, 611-622.

Caille, N., Swanwick, S., Zhao, F.J., McGrath, S.P. (2004) Arsenic hyperaccumulation by Pteris vittata from arsenic contaminated soils and the effect of liming and phosphate fertilisation. Environmental Pollution 132, 113-120.

Campbell, E.J., Schenk, P.M., Kazan, K., Penninckx, I.A.M.A., Anderson, J.P., Maclean, D.J., Cammue, B.P.A., Ebert, P.R., Manners, J.M. (2003) Pathogen-Responsive Expression

of a Putative ATP-Binding Cassette Transporter Gene Conferring Resistance to the Diterpenoid Sclareol Is Regulated by Multiple Defense Signaling Pathways in Arabidopsis. Plant Physiol 133, 1272-1284.

Campos, P., Semedo, J.N., Pais, I., Oliveira, M.M.R., Passarinho, J., Santos, M., Almeida, A., R Costa, A., Pinheiro, N., Bagorro, C., Coco, J., Costa, R., Coutinho, J., Maçãs, B. (2015) Physiological Responses to Drought in Four Developed Triticum aestivum Groups.

Cao, X., Ma, L.Q., Tu, C. (2004) Antioxidative responses to arsenic in the arsenic-hyperaccumulator Chinese brake fern (Pteris vittata L.). Environmental Pollution 128, 317-325.

Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., Knight, R. (2010a) PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26, 266-267.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R. (2010b) QIIME allows analysis of high-throughput community sequencing data. Nature methods 7, 335-336.

Carbonell-Barrachina, A.A., Aarabi, M.A., DeLaune, R.D., Gambrell, R.P., Patrick, W.H. (1998) The influence of arsenic chemical form and concentration on Spartina patens and Spartina alterniflora growth and tissue arsenic concentration. Plant and Soil 198, 33-43.

Carrasco-Gil, S., Estebaranz-Yubero, M., Medel-Cuesta, D., Millán, R., Hernández, L.E. (2012) Influence of nitrate fertilization on Hg uptake and oxidative stress parameters in alfalfa plants cultivated in a Hg-polluted soil. Environmental and Experimental Botany 75, 16-24.

Castanheira, N.L., Dourado, A.C., Pais, I., Semedo, J., Scotti-Campos, P., Borges, N., Carvalho, G., Barreto Crespo, M.T., Fareleira, P. (2017) Colonization and beneficial effects on annual ryegrass by mixed inoculation with plant growth promoting bacteria. Microbiological Research 198, 47-55.

Catarecha, P., Segura, M.D., Franco-Zorrilla, J.M., García-Ponce, B., Lanza, M., Solano, R., Paz-Ares, J., Leyva, A. (2007) A Mutant of the Arabidopsis Phosphate Transporter PHT1;1 Displays Enhanced Arsenic Accumulation. The Plant Cell 19, 1123-1133.

CEC, 2006. Thematic strategy for soil protection. COM(2006)231 Final, Commission of the European Communities, Brussels (22.9.2006).

Chain, E.P.o.C.i.t.F. (2009) Scientific Opinion on Arsenic in Food. EFSA Journal 7, 1351-n/a.

Chakrabarty, D., Trivedi, P.K., Misra, P., Tiwari, M., Shri, M., Shukla, D., Kumar, S., Rai, A., Pandey, A., Nigam, D., Tripathi, R.D., Tuli, R. (2009) Comparative transcriptome analysis of arsenate and arsenite stresses in rice seedlings. Chemosphere 74, 688-702.

Chang, S., Puryear, J., Cairney, J. (1993) A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11, 113-116.

Chang, Y.C., Nawata, A., Jung, K., Kikuchi, S. (2012) Isolation and characterization of an arsenate-reducing bacterium and its application for arsenic extraction from contaminated soil. J Ind Microbiol Biotechnol 39, 37-44.

Chaparro, J.M., Badri, D.V., Bakker, M.G., Sugiyama, A., Manter, D.K., Vivanco, J.M. (2013) Root exudation of phytochemicals in Arabidopsis follows specific patterns that are developmentally programmed and correlate with soil microbial functions. PLoS One 8, e55731.

Chapman, P.M. (2012) Adaptive monitoring based on ecosystem services. Science of The Total Environment 415, 56-60.

Che-Castaldo, J.P., Inouye, D.W. (2015) Interspecific competition between a non-native metal-hyperaccumulating plant (Noccaea caerulescens, Brassicaceae) and a native congener across a soil-metal gradient. Australian Journal of Botany 63, 141-151.

Chen, J., He, F., Zhang, X., Sun, X., Zheng, J., Zheng, J. (2014) Heavy metal pollution decreases microbial abundance, diversity and activity within particle-size fractions of a paddy soil. FEMS Microbiol Ecol 87, 164-181.

Chibuike, G.U., Obiora, S. (2014) Heavy Metal Polluted Soils: Effect on Plants and Bioremediation Methods. Applied and Environmental Soil Science, vol. 2014, 1-12.

Chung, K.T., Wong, T.Y., Wei, C.I., Huang, Y.W., Lin, Y. (1998) Tannins and human health: a review. Crit Rev Food Sci Nutr 38, 421-464.

Clark, G.T., Dunlop, J., Phung, H.T. (2000) Phosphate absorption by Arabidopsis thaliana: interactions between phosphorus status and inhibition by arsenate. Functional Plant Biology 27, 959-965.

Clemens, S. (2006) Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. Biochimie 88, 1707-1719.

Clemens, S., Peršoh, D. (2009) Multi-tasking phytochelatin synthases. Plant Science 177, 266-271.

CMPA Consejería de Medio Ambiente del Principado de Asturias (regional Ministry of the Environment of the Principality of Asturias), 2001. Inventario y caracterización de suelos contaminados del Principado de Asturias. Dirección General de Calidad Ambiental. Expediente AT/2000/58-97. Oviedo, Spain.

Coats, V.C., Rumpho, M.E. (2014) The rhizosphere microbiota of plant invaders: an overview of recent advances in the microbiomics of invasive plants. Frontiers in Microbiology 5, 368-368.

Cobbett, C. S. (2000). Phytochelatins and Their Roles in Heavy Metal Detoxification. Plant Physiology 123(3): 825-832.

Cobbett, C., Goldsbrough, P. (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53, 159-182.

Cobbett, C.S. (2000) Phytochelatins and Their Roles in Heavy Metal Detoxification. Plant Physiol 123, 825-832.

Cobbett, C.S., May, M.J., Howden, R., Rolls, B. (1998) The glutathione-deficient, cadmium-sensitive mutant, cad2-1, of Arabidopsis thaliana is deficient in gamma-glutamylcysteine synthetase. Plant J 16, 73-78.

Commission, European. 2000. Ambient Air Pollution by AS, CD and NI Compounds (Position Paper—Final), Luxembourg: Office for Official Publications of the European Communities.

Compant, S., Clément, C., Sessitsch, A. (2010) Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry 42, 669-678.

Conrath, U., Beckers, G.J., Flors, V., Garcia-Agustin, P., Jakab, G., Mauch, F., Newman, M.A., Pieterse, C.M., Poinssot, B., Pozo, M.J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L., Mauch-Mani, B. (2006) Priming: getting ready for battle. Mol Plant Microbe Interact 19, 1062-1071.

Contesto, C., Desbrosses, G., Lefoulon, C., Béna, G., Borel, F., Galland, M., Gamet, L., Varoquaux, F., Touraine, B. (2008) Effects of rhizobacterial ACC deaminase activity on Arabidopsis indicate that ethylene mediates local root responses to plant growth-promoting rhizobacteria. Plant Science 175, 178-189.

Cordos, E.A., Frentiua, T., Pontaa, M., Marginean, l., Abrahamb, B., Roman, C. (2006) Distribution study of inorganic arsenic (III) and (V) species in soil and their mobility in the area of Baia-Mare, Romania. Chemical Speciation & Bioavailability 18, 11-25.

Corsini, A., Cavalca, L., Crippa, L., Zaccheo, P., Andreoni, V. (2010) Impact of glucose on microbial community of a soil containing pyrite cinders: Role of bacteria in arsenic mobilization under submerged condition. Soil Biology and Biochemistry 42, 699-707.

Croes, S., Weyens, N., Janssen, J., Vercampt, H., Colpaert, J.V., Carleer, R., Vangronsveld, J. (2013) Bacterial communities associated with Brassica napus L. grown on trace element-contaminated and non-contaminated fields: a genotypic and phenotypic comparison. Microb Biotechnol 6, 371-384.

Cuypers, A., Hendrix, S., Amaral Dos Reis, R., De Smet, S., Deckers, J., Gielen, H., Jozefczak, M., Loix, C., Vercampt, H., Vangronsveld, J., Keunen, E. (2016) Hydrogen Peroxide, Signaling in Disguise during Metal Phytotoxicity. Front Plant Sci 7, 470-470.

Czech, V., Czövek, P., Fodor, J., Bóka, K., Fodor, F. (2008) Investigation of arsenate phytotoxicity in cucumber plants.

Dahmani-Muller, H., van Oort, F., Gélie, B., Balabane, M. (2000) Strategies of heavy metal uptake by three plant species growing near a metal smelter. Environmental Pollution 109, 231-238.

Dai, F., Qiu, L., Ye, L., Wu, D., Zhou, M., Zhang, G. (2011) Identification of a phytase gene in barley (Hordeum vulgare L.). PLoS One 6, e18829-e18829.

DalCorso, G., Farinati, S., Furini, A. (2010) Regulatory networks of cadmium stress in plants. Plant signaling & behavior 5, 663-667.

Das, I. (2016) Arsenic mitigation in soil-plant system through zinc application in West Bengal soils. Bioremediation journal v. 20, pp. 24-37-2016 v.2020 no.2011.

Das, I., Sanyal, S. K., Koushik G., and Das, D. K.. (2016). Arsenic mitigation in soil-plant system through zinc application in West Bengal soils. Bioremediation journal, 20: 24-37.

Das, K., Roychoudhury, A. (2014) Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Frontiers in Environmental Science 2.

Dauch, A.L., Jabaji-Hare, S.H. (2006) Metallothionein and bZIP Transcription Factor Genes from Velvetleaf and Their Differential Expression Following Colletotrichum coccodes Infection. Phytopathology 96, 1116-1123.

Dave, R., Singh, P.K., Tripathi, P., Shri, M., Dixit, G., Dwivedi, S., Chakrabarty, D., Trivedi, P.K., Sharma, Y.K., Dhankher, O.P., Corpas, F.J., Barroso, J.B., Tripathi, R.D. (2013) Arsenite tolerance is related to proportional thiolic metabolite synthesis in rice (Oryza sativa L.). Arch Environ Contam Toxicol 64, 235-242.

Davis, M.A., Pritchard, S.G., Boyd, R.S., Prior, S.A. (2001) Developmental and induced responses of nickel-based and organic defences of the nickel-hyperaccumulating shrub, Psychotria douarrei. New Phytologist 150, 49-58.

De la Rosa, G., Parsons, J.G., Martinez-Martinez, A., Peralta-Videa, J.R., Gardea-Torresdey, J.L. (2006) Spectroscopic study of the impact of arsenic speciation on arsenic/phosphorus uptake and plant growth in tumbleweed (Salsola kali). Environmental Science & Technology 40, 1991-1996.

De Mandal, S., Panda, A. (2015) Microbial Ecology in the Era of Next Generation Sequencing. Next Generat Sequencing & Applications, S1: 001.

del Pozo, J.C., Allona, I., Rubio, V., Leyva, A., de la Pena, A., Aragoncillo, C., Paz-Ares, J. (1999) A type 5 acid phosphatase gene from Arabidopsis thaliana is induced by phosphate starvation and by some other types of phosphate mobilising/oxidative stress conditions. Plant J 19, 579-589.

Delatorre, C., Rodríguez, A., Rodríguez, L., Majada, J.P., Ordás, R.J., Feito, I. (2017) Hormonal profiling: Development of a simple method to extract and quantify phytohormones in complex matrices by UHPLC–MS/MS. Journal of Chromatography B 1040, 239-249.

Deletoile, A., Decre, D., Courant, S., Passet, V., Audo, J., Grimont, P., Arlet, G., Brisse, S. (2009) Phylogeny and identification of Pantoea species and typing of Pantoea agglomerans strains by multilocus gene sequencing. J Clin Microbiol 47, 300-310.

Demiral, T., Türkan, İ. (2005) Comparative lipid peroxidation, antioxidant defense systems and proline content in roots of two rice cultivars differing in salt tolerance. Environmental and Experimental Botany 53, 247-257.

Dennis, P.G., Miller, A.J., Hirsch, P.R. (2010) Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? FEMS Microbiol Ecol 72, 313-327.

Depuydt, S., Trenkamp, S., Fernie, A.R., Elftieh, S., Renou, J.-P., Vuylsteke, M., Holsters, M., Vereecke, D. (2009) An integrated genomics approach to define niche establishment by Rhodococcus fascians. Plant Physiol 149, 1366-1386.

Dhankher, O. P., Y. Li, B. P. Rosen, J. Shi, D. Salt, J. F. Senecoff, N. A. Sashti and R. B. Meagher (2002). Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and gamma-glutamylcysteine synthetase expression. Nat Biotechnol 20(11): 1140-1145.

Dhankher, O.P., Li, Y., Rosen, B.P., Shi, J., Salt, D., Senecoff, J.F., Sashti, N.A., Meagher, R.B. (2002) Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and gamma-glutamylcysteine synthetase expression. Nat Biotechnol 20, 1140-1145.

Dhankher, O.P., Rosen, B.P., McKinney, E.C., Meagher, R.B. (2006) Hyperaccumulation of arsenic in the shoots of Arabidopsis silenced for arsenate reductase (ACR2). Proceedings of the National Academy of Sciences of the United States of America 103, 5413-5418.

Dhankher, O.P., Rosen, B.P., McKinney, E.C., Meagher, R.B. (2006) Hyperaccumulation of arsenic in the shoots of Arabidopsis silenced for arsenate reductase (ACR2). Proceedings of the National Academy of Sciences of the United States of America 103, 5413-5418.

Dhariwal, A., Chong, J., Habib, S., King, I.L., Agellon, L.B., Xia, J. (2017) MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic Acids Res 45, W180-w188.

Dickinson, N.M., Pulford, I.D. (2005) Cadmium phytoextraction using short-rotation coppice Salix: the evidence trail. Environ Int 31, 609-613.

Drewniak, L., Sklodowska, A. (2013) Arsenic-transforming microbes and their role in biomining processes. Environ Sci Pollut Res Int 20, 7728-7739.

Droux, M. (2004) Sulfur assimilation and the role of sulfur in plant metabolism: a survey. Photosynth Res 79, 331-348.

Du, D., van Veen, H.W., Murakami, S., Pos, K.M., Luisi, B.F. (2015) Structure, mechanism and cooperation of bacterial multidrug transporters. Current Opinion in Structural Biology 33, 76-91.

Duan, G.L., Zhou, Y., Tong, Y.P., Mukhopadhyay, R., Rosen, B.P., Zhu, Y.G. (2007) A CDC25 homologue from rice functions as an arsenate reductase. New Phytol 174, 311-321.

Duan, X., Jiang, Y., Su, X., Zhang, Z., Shi, J. (2007) Antioxidant properties of anthocyanins extracted from litchi (Litchi chinenesis Sonn.) fruit pericarp tissues in relation to their role in the pericarp browning. Food Chemistry 101, 1365-1371.

Duncan, E.G., Maher, W.A., Foster, S.D., Krikowa, F., O'Sullivan, C.A., Roper, M.M. (2017) Dimethylarsenate (DMA) exposure influences germination rates, arsenic uptake and arsenic species formation in wheat. Chemosphere 181, 44-54.

Dutta, P., Mondal, S. (2014) Changes in pigments and photosynthetic parameters of cowpea under two inorganic arsenicals.

Dzionek, A., Wojcieszyńska, D., Guzik, U. (2016) Natural carriers in bioremediation: A review. Electronic Journal of Biotechnology 23, 28-36.

East, R. (2013) Microbiome: Soil science comes to life. Nature 501, S18.

EC. 2006. Communication from the Commission to the Council, the European Parliament, the European Economic and Social Committee and the Committee of the Regions - Thematic Strategy for Soil Protection [SEC(2006)620] [SEC(2006)1165].

Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460-2461.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194-2200.

Eevers, N., Gielen, M., Sánchez-López, A., Jaspers, S., White, J.C., Vangronsveld, J., Weyens, N. (2015) Optimization of isolation and cultivation of bacterial endophytes through addition of plant extract to nutrient media. Microb Biotechnol 8, 707-715.

Ehlrich, H.L. (1990) Geomicrobiology. Marcel Dekker New York.

Ellis, D.R., Gumaelius, L., Indriolo, E., Pickering, I.J., Banks, J.A., Salt, D.E. (2006) A novel arsenate reductase from the arsenic hyperaccumulating fern Pteris vittata. Plant Physiol 141, 1544-1554.

Ellman, G.L. (1959) Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics 82, 70-77.

Emamverdian, A., Ding, Y., Mokhberdoran, F., Xie, Y. (2015) Heavy metal stress and some mechanisms of plant defense response. TheScientificWorldJournal 2015, 756120-756120.

Evangelou, M.W., Robinson, B.H., Gunthardt-Goerg, M.S., Schulin, R. (2013) Metal uptake and allocation in trees grown on contaminated land: implications for biomass production. Int J Phytoremediation 15, 77-90.

Fageria, N.K., Baligar, V.C., (2005) NUTRIENT AVAILABILITY, in: Hillel, D. (Ed.), Encyclopedia of Soils in the Environment. Elsevier, Oxford, pp. 63-71.

Fahey, R.C. (2013) Glutathione analogs in prokaryotes. Biochim Biophys Acta 1830, 3182-3198.

Farooq, M.A., Gill, R.A., Islam, F., Ali, B., Liu, H., Xu, J., He, S., Zhou, W. (2016) Methyl Jasmonate Regulates Antioxidant Defense and Suppresses Arsenic Uptake in Brassica napus L. Front Plant Sci 7, 468-468.

Farrar, K., Bryant, D., Cope-Selby, N. (2014) Understanding and engineering beneficial plant-microbe interactions: plant growth promotion in energy crops. Plant biotechnology journal 12, 1193-1206.

Favas, P., Pratas, J., Varun, M., D'Souza, R., Paul, M.S., (2014) Phytoremediation of Soils Contaminated with Metals and Metalloids at Mining Areas: Potential of Native Flora. in: Hernandez-Soriano, M.C. (Ed.), Environmental Risk Assessment of Soil Contamination, InTech.

Fayiga, A.O., Saha, U.K. (2016) Arsenic hyperaccumulating fern: Implications for remediation of arsenic contaminated soils. Geoderma 284, 132-143.

Fernandes, A.P., Holmgren, A. (2004) Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. Antioxid Redox Signal 6, 63-74.

Fernandez, R., A. Bertrand, R. Reis, M. P. Mourato, L. L. Martins and A. Gonzalez (2013). Growth and physiological responses to cadmium stress of two populations of Dittrichia viscosa (L.) Greuter. J Hazard Mater 244-245: 555-562.

Fernandez, R., Bertrand, A., Casares, A., Garcia, R., Gonzalez, A., Tames, R.S. (2008) Cadmium accumulation and its effect on the in vitro growth of woody fleabane and mycorrhized white birch. Environmental Pollution 152, 522-529.

Fernández, R., Bertrand, A., García, J.I., Tamés, R.S., González, A. (2012) Lead accumulation and synthesis of non-protein thiolic peptides in selected clones of Melilotus alba and Melilotus officinalis. Environmental and Experimental Botany 78, 18-24.

Fernandez, R., Bertrand, A., Reis, R., Mourato, M.P., Martins, L.L., Gonzalez, A. (2013) Growth and physiological responses to cadmium stress of two populations of Dittrichia viscosa (L.) Greuter. J Hazard Mater 244-245, 555-562.

Fernández, R., Carballo, I., Nava, H., Sánchez-Tamés, R., Bertrand, A., González, A. (2010) Looking for native hyperacumulator species useful in phytoremediation. In: Handbook of Phytoremediation. Golubev, I.A (ed), Nova Science Publishers, Inc.New York, pp.297-330.

Fernandez, R., Fernandez-Fuego, D., Bertrand, A., Gonzalez, A. (2014) Strategies for Cd accumulation in Dittrichia viscosa (L.) Greuter: role of the cell wall, non-protein thiols and organic acids. Plant Physiol Biochem 78, 63-70.

Fernandez-Fuego, D., Bertrand, A., Gonzalez, A. (2017) Metal accumulation and detoxification mechanisms in mycorrhizal Betula pubescens. Environmental Pollution 231, 1153-1162.

Fernandez-Navarro, P., Garcia-Perez, J., Ramis, R., Boldo, E., Lopez-Abente, G. (2012) Proximity to mining industry and cancer mortality. Sci Total Environ 435-436, 66-73.

Fierer, N., Jackson, R.B. (2006) The diversity and biogeography of soil bacterial communities. Proc Natl Acad Sci U S A 103, 626-631.

Fierer, N., Lauber, C.L., Zhou, N., McDonald, D., Costello, E.K., Knight, R. (2010) Forensic identification using skin bacterial communities. Proceedings of the National Academy of Sciences 107, 6477-6481.

Finnegan, P. M. and W. Chen (2012). Arsenic Toxicity: The Effects on Plant Metabolism. Frontiers in Physiology 3: 182.

Fitz, W.J., Wenzel, W.W. (2002) Arsenic transformations in the soil–rhizosphere–plant system: fundamentals and potential application to phytoremediation. Journal of Biotechnology 99, 259-278.

Flora, S.J., Bhadauria, S., Kannan, G.M., Singh, N. (2007) Arsenic induced oxidative stress and the role of antioxidant supplementation during chelation: a review. J Environ Biol 28, 333-347.

Fu, S.F., Chen, P.Y., Nguyen, Q.T., Huang, L.Y., Zeng, G.R., Huang, T.L., Lin, C.Y., Huang, H.J. (2014) Transcriptome profiling of genes and pathways associated with arsenic toxicity and tolerance in Arabidopsis. BMC Plant Biol 14, 94.

Furini, A., Manara, A., DalCorso, G. (2015) Editorial: Environmental phytoremediation: plants and microorganisms at work. Front Plant Sci 6, 520-520.

Gajić, G., Djurdjević, L., Kostić, O., Jarić, S., Mitrović, M., Pavlović, P. (2018) Ecological Potential of Plants for Phytoremediation and Ecorestoration of Fly Ash Deposits and Mine Wastes. Frontiers in Environmental Science 6.

Gallego, J.R., Esquinas, N., Rodríguez-Valdés, E., Menéndez-Aguado, J.M., Sierra, C. (2015) Comprehensive waste characterization and organic pollution co-occurrence in a Hg and As mining and metallurgy brownfield. Journal of Hazardous Materials 300, 561-571.

Gallego, J.R., Rodríguez-Valdés, E., Esquinas, N., Fernández-Braña, A., Afif, E. (2016) Insights into a 20-ha multi-contaminated brownfield megasite: An environmental forensics approach. Science of The Total Environment 563, 683-692.

Garbeva, P., Van Veen, J.A., van Elsas, J. (2004) Microbial diversity in soil: Selection of Microbial Populations by Plant and Soil Type and Implications for Disease Suppressiveness.

Garbinski, L.D., Rosen, B.P., Chen, J. (2019) Pathways of arsenic uptake and efflux. Environ Int 126, 585-597.

George, T.S., Richardson, A.E., Li, S.S., Gregory, P.J., Daniell, T.J. (2009) Extracellular release of a heterologous phytase from roots of transgenic plants: does manipulation of

rhizosphere biochemistry impact microbial community structure? FEMS Microbiol Ecol 70, 433-445.

Ghosh, P., Rathinasabapathi, B., Ma, L.Q. (2011) Arsenic-resistant bacteria solubilized arsenic in the growth media and increased growth of arsenic hyperaccumulator Pteris vittata L. Bioresource Technology 102, 8756-8761.

Ghosh, P., Rathinasabapathi, B., Teplitski, M., Ma, L.Q. (2015) Bacterial ability in AsIII oxidation and AsV reduction: Relation to arsenic tolerance, P uptake, and siderophore production. Chemosphere 138, 995-1000.

Gill, R.A., Zang, L., Ali, B., Farooq, M.A., Cui, P., Yang, S., Ali, S., Zhou, W. (2015) Chromium-induced physio-chemical and ultrastructural changes in four cultivars of Brassica napus L. Chemosphere 120, 154-164.

Glick, B.R. (2012) Plant Growth-Promoting Bacteria: Mechanisms and Applications. Scientifica 2012, 15.

Glick, B.R., Penrose, D.M., Li, J. (1998) A Model For the Lowering of Plant Ethylene Concentrations by Plant Growth-promoting Bacteria. Journal of Theoretical Biology 190, 63-68.

Gokulan, K., Arnold, M.G., Jensen, J., Vanlandingham, M., Twaddle, N.C., Doerge, D.R., Cerniglia, C.E., Khare, S. (2018) Exposure to Arsenite in CD-1 Mice during Juvenile and Adult Stages: Effects on Intestinal Microbiota and Gut-Associated Immune Status. mBio 9, e01418-01418.

Gong, J. M., D. A. Lee and J. I. Schroeder (2003). "Long-distance root-to-shoot transport of phytochelatins and cadmium in Arabidopsis." Proc Natl Acad Sci U S A 100(17): 10118-10123

Gonzalez, E., Brereton, N.J., Marleau, J., Guidi Nissim, W., Labrecque, M., Pitre, F.E., Joly, S. (2015) Meta-transcriptomics indicates biotic cross-tolerance in willow trees cultivated on petroleum hydrocarbon contaminated soil. BMC Plant Biol 15, 246.

González-Guerrero, M., Escudero, V., Saéz, Á., Tejada-Jiménez, M. (2016) Transition Metal Transport in Plants and Associated Endosymbionts: Arbuscular Mycorrhizal Fungi and Rhizobia. Front Plant Sci 7.

Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., Rokhsar, D.S. (2012) Phytozome: a comparative platform for green plant genomics. Nucleic Acids Res 40, D1178-D1186.

Govarthanan, M., Lee, S.M., Kamala-Kannan, S., Oh, B.T. (2015) Characterization, real-time quantification and in silico modeling of arsenate reductase (arsC) genes in arsenic-resistant Herbaspirillum sp. GW103. Res Microbiol 166, 196-204.

Gozzo, F. (2003) Systemic acquired resistance in crop protection: from nature to a chemical approach. J Agric Food Chem 51, 4487-4503.

Graham, R.D., Stangoulis, J.C.R. (2003) Trace Element Uptake and Distribution in Plants. The Journal of Nutrition 133, 1502S-1505S.

Greger, M., Landberg, T. (1999) Use of Willow in Phytoextraction. International Journal of Phytoremediation 1, 115-123.

Grennan, A.K. (2011) Metallothioneins, a diverse protein family. Plant Physiol 155, 1750-1751.

Groppa, M.D., Benavides, M.P., Zawoznik, M.S. (2012) Root hydraulic conductance, aquaporins and plant growth promoting microorganisms: A revision. Applied Soil Ecology 61, 247-254.

Guarino, C., Paura, B., Sciarrillo, R. (2018) Enhancing Phytoextraction of HMs at Real Scale, by Combining Salicaceae Trees With Microbial Consortia. Frontiers in Environmental Science 6.

Gurevich, A., Saveliev, V., Vyahhi, N., Tesler, G. (2013) QUAST: quality assessment tool for genome assemblies. Bioinformatics 29, 1072-1075.

Hale, K.L., McGrath, S.P., Lombi, E., Stack, S.M., Terry, N., Pickering, I.J., George, G.N., Pilon-Smits, E.A. (2001) Molybdenum sequestration in Brassica species. A role for anthocyanins? Plant Physiol 126, 1391-1402.

Hall, J. (2002) Cellular mechanisms for heavy metal detoxification and tolerance. Journal of Experimental Botany 53, 1-11.

Hamamura, N., Itai, T., Liu, Y., Reysenbach, A.L., Damdinsuren, N., Inskeep, W.P. (2014) Identification of anaerobic arsenite-oxidizing and arsenate-reducing bacteria associated with an alkaline saline lake in Khovsgol, Mongolia. Environ Microbiol Rep 6, 476-482.

Hammer, D., Kayser, A., Keller, C. (2003) Phytoextraction of Cd and Zn with Salix viminalis in field trials. Soil Use and Management 19, 187-192.

Han, Y.-H., Fu, J.-W., Chen, Y., Rathinasabapathi, B., Ma, L.Q. (2016) Arsenic uptake, arsenite efflux and plant growth in hyperaccumulator Pteris vittata: Role of arsenic-resistant bacteria. Chemosphere 144, 1937-1942.

Hardoim, P.R., van Overbeek, L.S., Berg, G., Pirttilä, A.M., Compant, S., Campisano, A., Döring, M., Sessitsch, A. (2015) The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. Microbiology and molecular biology reviews : MMBR 79, 293-320.

Hardoim, P.R., van Overbeek, L.S., Berg, G., Pirttilä, A.M., Compant, S., Campisano, A., Döring, M., Sessitsch, A. (2015) The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. Microbiology and molecular biology reviews : MMBR 79, 293-320.

Hardoim, P.R., van Overbeek, L.S., Elsas, J.D. (2008) Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol 16, 463-471.

Harter, R.D. (1983) Effect of Soil pH on Adsorption of Lead, Copper, Zinc, and Nickel1. Soil Science Society of America Journal 47, 47-51.DalCorso, G., Manara, A., Furini, A. (2013) An overview of heavy metal challenge in plants: from roots to shoots. Metallomics 5, 1117-1132.

Harter, R.D., Naidu, R. (2001) An Assessment of Environmental and Solution Parameter Impact on Trace-Metal Sorption by Soils Scientific Contribution No. 2012 from the New Hampshire Agricultural Experiment Station. Soil Science Society of America Journal 65, 597-612.

Hartley-Whitaker, J., Ainsworth, G., Meharg, A.A. (2001a) Copper- and arsenate-induced oxidative stress in Holcus lanatus L. clones with differential sensitivity. Plant Cell Environ 24, 713-722.

Hartley-Whitaker, J., Ainsworth, G., Vooijs, R., Bookum, W.T., Schat, H., Meharg, A.A. (2001b) Phytochelatins Are Involved in Differential Arsenate Tolerance in Holcus lanatus. Plant Physiol 126, 299-306.

Hartmann, A., Schmid, M., Tuinen, D.v., Berg, G. (2009) Plant-driven selection of microbes. Plant and Soil 321, 235-257.

Hasan, M.K., Cheng, Y., Kanwar, M.K., Chu, X.-Y., Ahammed, G.J., Qi, Z.-Y. (2017) Responses of Plant Proteins to Heavy Metal Stress-A Review. Front Plant Sci 8, 249-268.

Hasanuzzaman, M., K. Nahar, T. I. Anee and M. Fujita (2017). Glutathione in plants: biosynthesis and physiological role in environmental stress tolerance. Physiology and molecular biology of plants: an international journal of functional plant biology 23(2): 249-268.

Hawkes, C.V., DeAngelis, K.M., Firestone, M.K., (2007) CHAPTER 1 - Root Interactions with Soil Microbial Communities and Processes, in: Cardon, Z.G., Whitbeck, J.L. (Eds.), The Rhizosphere. Academic Press, Burlington, pp. 1-29.

Herath, I., Vithanage, M., Bundschuh, J., Maity, J.P., Bhattacharya, P. (2016) Natural Arsenic in Global Groundwaters: Distribution and Geochemical Triggers for Mobilization. Current Pollution Reports 2, 68-89.

Herbette, S., Taconnat, L., Hugouvieux, V., Piette, L., Magniette, M.L., Cuine, S., Auroy, P., Richaud, P., Forestier, C., Bourguignon, J., Renou, J.P., Vavasseur, A., Leonhardt, N. (2006) Genome-wide transcriptome profiling of the early cadmium response of Arabidopsis roots and shoots. Biochimie 88, 1751-1765.

Hermle, S., Günthardt-Goerg, M.S., Schulin, R. (2006) Effects of metal-contaminated soil on the performance of young trees growing in model ecosystems under field conditions. Environmental Pollution 144, 703-714.

Hernández-Prieto, M.A., Semeniuk, T.A., Futschik, M.E. (2014) Toward a systems-level understanding of gene regulatory, protein interaction, and metabolic networks in cyanobacteria. Frontiers in genetics 5, 191-191.

Hiltner, L. (1904) Über neuere erfahrungen und probleme auf dem gebiete der bodenbakteriologie unter bessonderer berücksichtigung der gründung und brache. Arb. Dtsch. Landwirtsch. Ges. Berl. 98 59–78.

Hinsinger, P., Bengough, A.G., Vetterlein, D., Young, I.M. (2009) Rhizosphere: biophysics, biogeochemistry and ecological relevance. Plant and Soil 321, 117-152.

Hinsinger, P., Brauman, A., Devau, N., Gérard, F., Jourdan, C., Laclau, J.-P., Le Cadre, E., Jaillard, B., Plassard, C. (2011) Acquisition of phosphorus and other poorly mobile nutrients by roots. Where do plant nutrition models fail? Plant and Soil 348, 29.

Hirata, K., Tsuji, N., Miyamoto, K. (2005) Biosynthetic regulation of phytochelatins, heavy metal-binding peptides. J Biosci Bioeng 100, 593-599.

Hirsch, P.R., Mauchline, T.H. (2012) Who is who in the plant root microbiome? Nat Biotechnol 30, 961.

Hirschi, K.D., Korenkov, V.D., Wilganowski, N.L., Wagner, G.J. (2000) Expression of arabidopsis CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. Plant Physiol 124, 125-133.

Hoeft, S.E., Kulp, T.R., Han, S., Lanoil, B., Oremland, R.S. (2010) Coupled arsenotrophy in a hot spring photosynthetic biofilm at Mono Lake, California. Appl Environ Microbiol 76, 4633-4639.

Hokura, A., Omuma, R., Terada, Y., Kitajima, N., Abe, T., Saito, H., Yoshida, S., Nakai, I. (2006) Arsenic distribution and speciation in an arsenic hyperaccumulator fern by X-ray spectrometry utilizing a synchrotron radiation source.

Holford, I. (1997) Soil phosphorus: its measurement, and its uptake by plants. Soil Research 35, 227-240.

Hooda, P. (2010) Trace Elements in Soils. Wiley-Blackwell, pp.616.

Hosseini, M.-J., Shaki, F., Ghazi-Khansari, M., Pourahmad, J. (2013) Toxicity of Arsenic (III) on Isolated Liver Mitochondria: A New Mechanistic Approach. Iranian journal of pharmaceutical research : IJPR 12, 121-138.

Howden, R., Andersen, C.R., Goldsbrough, P.B., Cobbett, C.S. (1995) A cadmium-sensitive, glutathione-deficient mutant of Arabidopsis thaliana. Plant Physiol 107, 1067-1073.

Hu, M., Li, F., Liu, C., Wu, W. (2015) The diversity and abundance of As(III) oxidizers on root iron plaque is critical for arsenic bioavailability to rice. Scientific Reports 5, 13611-13611.

Huang, J.H., Fecher, P., Ilgen, G., Hu, K.N., Yang, J. (2012a) Speciation of arsenite and arsenate in rice grain – Verification of nitric acid based extraction method and mass sample survey. Food Chemistry 130, 453-459.

Huang, T.-L., Nguyen, Q.T.T., Fu, S.-F., Lin, C.-Y., Chen, Y.-C., Huang, H.-J. (2012b) Transcriptomic changes and signalling pathways induced by arsenic stress in rice roots. Plant Mol Biol 80, 587-608.

Huang, Y., Hatayama, M., Inoue, C. (2011) Characterization of As efflux from the roots of As hyperaccumulator Pteris vittata L. Planta 234, 1275-1284.

Hughes, M.F. (2002) Arsenic toxicity and potential mechanisms of action. Toxicol Lett 133, 1-16.

Hunter, P., Teakle, G., Bending, G. (2014) Root traits and microbial community interactions in relation to phosphorus availability and acquisition, with particular reference to Brassica. Front Plant Sci 5.

Isayenkov, S.V., Maathuis, F.J. (2008) The Arabidopsis thaliana aquaglyceroporin AtNIP7;1 is a pathway for arsenite uptake. FEBS Lett 582, 1625-1628.

Jacob, J.M., Karthik, C., Saratale, R.G., Kumar, S.S., Prabakar, D., Kadirvelu, K., Pugazhendhi, A. (2018) Biological approaches to tackle heavy metal pollution: A survey of literature. J Environ Manage 217, 56-70.

Jacoby, R., Peukert, M., Succurro, A., Koprivova, A., Kopriva, S. (2017) The Role of Soil Microorganisms in Plant Mineral Nutrition-Current Knowledge and Future Directions. Front Plant Sci 8, 1617-1617.

Jakobsen, I., Gavito, M.E., Curtis, P.S., Mikkelsen, T.N. (2001) Interactive effects of soil temperature, atmospheric carbon dioxide and soil N on root development, biomass and nutrient uptake of winter wheat during vegetative growth. Journal of Experimental Botany 52, 1913-1923.

Janssen, J., Weyens, N., Croes, S., Beckers, B., Meiresonne, L., Van Peteghem, P., Carleer, R., Vangronsveld, J. (2015) Phytoremediation of Metal Contaminated Soil Using Willow: Exploiting Plant-Associated Bacteria to Improve Biomass Production and Metal Uptake. Int J Phytoremediation 17, 1123-1136.

Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M., Sait, M. (2002) Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. Appl Environ Microbiol 68, 2391-2396.

Jelenska, J., van Hal, J.A., Greenberg, J.T. (2010) Pseudomonas syringae hijacks plant stress chaperone machinery for virulence. Proceedings of the National Academy of Sciences of the United States of America 107, 13177-13182.

Jensen, J.K., Holm, P.E., Nejrup, J., Larsen, M.B., Borggaard, O.K. (2009) The potential of willow for remediation of heavy metal polluted calcareous urban soils. Environmental Pollution 157, 931-937.

Ji, R., L. Zhou, J. Liu, Y. Wang, L. Yang, Q. Zheng, C. Zhang, B. Zhang, H. Ge, Y. Yang, F. Zhao, S. Luan, and W. Lan. (2017). Calcium-dependent protein kinase CPK31 interacts with arsenic transporter AtNIP1;1 and regulates arsenite uptake in Arabidopsis thaliana, PLoS One, 12: e0173681.

Jiang, Y., Lei, M., Duan, L., Longhurst, P. (2015) Integrating phytoremediation with biomass valorisation and critical element recovery: A UK contaminated land perspective. Biomass and Bioenergy 83, 328-339.

Jorquera, M., Martinez, O., Maruyama, F., Marschner, P., de la Luz Mora, M. (2008) Current and future biotechnological applications of bacterial phytases and phytase-producing bacteria. Microbes Environ 23, 182-191.

Jozefczak, M., Remans, T., Vangronsveld, J., Cuypers, A. (2012) Glutathione is a key player in metal-induced oxidative stress defenses. International Journal of Molecular Sciences 13, 3145-3175.

Kabata-Pendias, (2010) Trace Elements in Soils and Plants, Fourth Edition. CRC Press, pp. 407-505.

Kang, J., Park, J., Choi, H., Burla, B., Kretzschmar, T., Lee, Y., Martinoia, E. (2011) Plant ABC Transporters. The Arabidopsis Book / American Society of Plant Biologists 9, e0153.

Kangatharalingam, N., Pierce, M.L., Bayles, M.B., Essenberg, M. (2002) Epidermal anthocyanin production as an indicator of bacterial blight resistance in cotton. Physiological and Molecular Plant Pathology 61, 189-195.

Kanwar, M.K., Poonam, Bhardwaj, R. (2015) Arsenic induced modulation of antioxidative defense system and brassinosteroids in Brassica juncea L. Ecotoxicol Environ Saf 115, 119-125.

Kaur, S., Kamli, M.R., Ali, A. (2011) Role of arsenic and its resistance in nature. Can J Microbiol 57, 769-774.

Kavanagh, K.L., Jörnvall, H., Persson, B., Oppermann, U. (2008) Medium- and short-chain dehydrogenase/reductase gene and protein families : the SDR superfamily: functional and structural diversity within a family of metabolic and regulatory enzymes. Cell Mol Life Sci 65, 3895-3906.

Kazakou, E., Dimitrakopoulos, P.G., Baker, A.J.M., Reeves, R.D., Troumbis, A.Y. (2008) Hypotheses, mechanisms and trade-offs of tolerance and adaptation to serpentine soils: from species to ecosystem level. Biological Reviews 83, 495-508.

Keesstra, S.D., Bouma, J., Wallinga, J., Tittonell, P., Smith, P., Cerdà, A., Montanarella, L., Quinton, J.N., Pachepsky, Y., van der Putten, W.H., Bardgett, R.D., Moolenaar, S., Mol, G., Jansen, B., Fresco, L.O. (2016) The significance of soils and soil science towards realization of the United Nations Sustainable Development Goals. SOIL 2, 111-128.

Keil, D.E., Berger-Ritchie, J., McMillin, G.A. (2011) Testing for Toxic Elements: A Focus on Arsenic, Cadmium, Lead, and Mercury. Laboratory Medicine 42, 735-742.

Kertulis, G.M., Ma, L.Q., MacDonald, G.E., Chen, R., Winefordner, J.D., Cai, Y. (2005) Arsenic speciation and transport in Pteris vittata L. and the effects on phosphorus in the xylem sap. Environmental and Experimental Botany 54, 239-247.

Keunen, E., Schellingen, K., Vangronsveld, J., Cuypers, A. (2016) Ethylene and Metal Stress: Small Molecule, Big Impact. Front Plant Sci 7.

Khalid, S., Shahid, M., Niazi, N.K., Rafiq, M., Bakhat, H.F., Imran, M., Abbas, T., Bibi, I., Dumat, C., (2017) Arsenic Behaviour in Soil-Plant System: Biogeochemical Reactions and Chemical Speciation Influences, in: Anjum, N.A., Gill, S.S., Tuteja, N. (Eds.), Enhancing Cleanup of Environmental Pollutants: Volume 2: Non-Biological Approaches. Springer International Publishing, Cham, pp. 97-140.

Khan, A.G., Kuek, C., Chaudhry, T.M., Khoo, C.S., Hayes, W.J. (2000) Role of plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation. Chemosphere 41, 197-207.

Kidd, P., Mench, M., Álvarez-López, V., Bert, V., Dimitriou, I., Friesl-Hanl, W., Herzig, R., Olga Janssen, J., Kolbas, A., Müller, I., Neu, S., Renella, G., Ruttens, A., Vangronsveld, J., Puschenreiter, M. (2015) Agronomic Practices for Improving Gentle Remediation of Trace Element-Contaminated Soils. International Journal of Phytoremediation 17, 1005-1037.

Kim, D.-Y., Bovet, L., Maeshima, M., Martinoia, E., Lee, Y. (2007) The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. The Plant Journal 50, 207-218.

Kim, D.-Y., Bovet, L., Maeshima, M., Martinoia, E., Lee, Y. (2007) The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. The Plant Journal 50, 207-218.

Kong, Z., Glick, B., (2017a) The Role of Bacteria in Phytoremediation: Innovations and Future Directions, pp. 327-353.

Kong, Z., Glick, B.R., (2017b) The Role of Plant Growth-Promoting Bacteria in Metal Phytoremediation, in: Poole, R.K. (Ed.), Advances in Microbial Physiology. Academic Press, pp. 97-132.

Konlechner, C., M. Türktaş, I. Langer, M. Vaculík, W. W. Wenzel, M. Puschenreiter and M.-T. Hauser (2013). Expression of zinc and cadmium responsive genes in leaves of willow (Salix caprea L.) genotypes with different accumulation characteristics. Environmental pollution (Barking, Essex : 1987) 178: 121-127.

Kosegarten, H., Koyro, H.-W. (2001) Apoplastic accumulation of iron in the epidermis of maize (Zea mays) roots grown in calcareous soil. Physiologia Plantarum 113, 515-522.

Kowalczyk, A., Latowski, D., (2018) Role of Plant-Microorganism Interactions in Plant Tolerance to Arsenic, in: Hasanuzzaman, M., Nahar, K., Fujita, M. (Eds.), Mechanisms of Arsenic Toxicity and Tolerance in Plants. Springer Singapore, Singapore, pp. 219-237.

Krishnamurthy, A., Rathinasabapathi, B. (2013) Auxin and its transport play a role in plant tolerance to arsenite-induced oxidative stress in Arabidopsis thaliana. Plant Cell Environ 36, 1838-1849.

Kroncke, K.D., Klotz, L.O. (2009) Zinc fingers as biologic redox switches? Antioxid Redox Signal 11, 1015-1027.

Krumins, J.A., Goodey, N.M., Gallagher, F. (2015) Plant–soil interactions in metal contaminated soils. Soil Biology and Biochemistry 80, 224-231.

Krupa, Z., Baranowska, M., Orzol, D. (1996) Can anthocyanins be considered as heavy metal indicator in higher plants? Acta Physiol Plant.

Kuffner, M., De Maria, S., Puschenreiter, M., Fallmann, K., Wieshammer, G., Gorfer, M., Strauss, J., Rivelli, A.R., Sessitsch, A. (2010) Culturable bacteria from Zn- and Cd-accumulating Salix caprea with differential effects on plant growth and heavy metal availability. J Appl Microbiol 108, 1471-1484.

Kumar, B., Smita, K., Cumbal Flores, L. (2017) Plant mediated detoxification of mercury and lead. Arabian Journal of Chemistry 10, S2335-S2342.

Kumar, S., Stecher, G., Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33, 1870-1874.

Kuppusamy, S., Palanisami, T., Mallavarapu, M., Kadiyala, V., Nadiu, R. (2016) In-situ remediation approaches for the management of contaminated sites: A comprehensive overview.

Kuzovkina, Y.A., Quigley, M.F. (2005) Willows Beyond Wetlands: Uses of Salix L. Species for Environmental Projects. Water, Air, and Soil Pollution 162, 183-204.

Laidlaw, W., Arndt, S., Huynh, T., Gregory, D., Baker, A. (2012) Phytoextraction of Heavy Metals by Willows Growing in Biosolids under Field Conditions.

Lampis, S., Santi, C., Ciurli, A., Andreolli, M., Vallini, G. (2015) Promotion of arsenic phytoextraction efficiency in the fern Pteris vittata by the inoculation of As-resistant bacteria: a soil bioremediation perspective. Front Plant Sci 6, 80.

Landberg, T., Greger, M. (2004) No phytochelatin (PC2 and PC3) detected in Salix viminalis. Physiologia Plantarum 121, 481-487.

Langille, M.G., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Vega Thurber, R.L., Knight, R., Beiko, R.G., Huttenhower, C. (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 31, 814-821.

Lauron-Moreau, A., Pitre, F.E., Argus, G.W., Labrecque, M., Brouillet, L. (2015) Phylogenetic relationships of american willows (Salix L., Salicaceae). PLoS One 10, e0121965.

Le Gall, H., F. Philippe, J. M. Domon, F. Gillet, J. Pelloux and C. Rayon (2015). "Cell Wall Metabolism in Response to Abiotic Stress." Plants (Basel) 4(1): 112-166.

LeBlanc, M.S., McKinney, E.C., Meagher, R.B., Smith, A.P. (2013) Hijacking membrane transporters for arsenic phytoextraction. Journal of Biotechnology 163, 1-9.

Levinson, W., Oppermann, H., Jackson, J. (1980) Transition series metals and sulfhydryl reagents induce the synthesis of four proteins in eukaryotic cells. Biochim Biophys Acta 606, 170-180.

Li, J., Jia, H., Han, X., Zhang, J., Sun, P., Lu, M., Hu, J. (2016) Selection of Reliable Reference Genes for Gene Expression Analysis under Abiotic Stresses in the Desert Biomass Willow, Salix psammophila. Front Plant Sci 7, 1505.

Li, J.H., Sun, Y.Y., Yin, Y., Ji, R., Wu, J.C., Wang, X.R., Guo, H.Y. (2010) Ethyl lactate-EDTA composite system enhances the remediation of the cadmium-contaminated soil by Autochthonous Willow (Salix x aureo-pendula CL 'J1011') in the lower reaches of the Yangtze River. Journal of Hazardous Materials 181, 673-678.

Li, J.T., Baker, A.J., Ye, Z.H., Wang, H.B., Shu, W.S. (2012) Phytoextraction of Cd-Contaminated Soils: Current Status and Future Challenges. Crit Rev Environ Sci Technol 42, 2113-2152.

Li, P., Lin, C., Cheng, H., Duan, X., Lei, K. (2015) Contamination and health risks of soil heavy metals around a lead/zinc smelter in southwestern China. Ecotoxicology and Environmental Safety 113, 391-399.

Li, R.-Y., Ago, Y., Liu, W.-J., Mitani, N., Feldmann, J., McGrath, S.P., Ma, J.F., Zhao, F.-J. (2009) The Rice Aquaporin Lsi1 Mediates Uptake of Methylated Arsenic Species. Plant Physiol 150, 2071-2080.

Li, W., Lan, P. (2017) The Understanding of the Plant Iron Deficiency Responses in Strategy I Plants and the Role of Ethylene in This Process by Omic Approaches. Front Plant Sci 8.

Li, Y., Dankher, O.P., Carreira, L., Smith, A.P., Meagher, R.B. (2006) The shoot-specific expression of gamma-glutamylcysteine synthetase directs the long-distance transport of thiol-peptides to roots conferring tolerance to mercury and arsenic. Plant Physiol 141, 288-298.

Li, Y., O. P. Dhankher, L. Carreira, D. Lee, A. Chen, J. I. Schroeder, R. S. Balish and R. B. Meagher (2004). "Overexpression of phytochelatin synthase in Arabidopsis leads to enhanced arsenic tolerance and cadmium hypersensitivity." Plant Cell Physiol 45(12): 1787-1797.

Lichtenthaler, H.K., Wellburn, A.R. (1983) Determinations of total carotenoids and chlorophylls of leaf extracts in different solvents. Biochemical Society Transactions 11, 591-592.

Lindsay, E.R., Maathuis, F.J.M. (2016) Arabidopsis thaliana NIP7;1 is involved in tissue arsenic distribution and tolerance in response to arsenate. FEBS Letters 590, 779-786.

Liu, C.-W., Murray, J.D. (2016) The Role of Flavonoids in Nodulation Host-Range Specificity: An Update. Plants (Basel) 5, 33.

Liu, W.-J., Wood, B.A., Raab, A., McGrath, S.P., Zhao, F.-J., Feldmann, J. (2010) Complexation of Arsenite with Phytochelatins Reduces Arsenite Efflux and Translocation from Roots to Shoots in Arabidopsis. Plant Physiol 152, 2211.

Liu, W.-J., Wood, B.A., Raab, A., McGrath, S.P., Zhao, F.-J., Feldmann, J. (2010) Complexation of Arsenite with Phytochelatins Reduces Arsenite Efflux and Translocation from Roots to Shoots in Arabidopsis. Plant Physiol 152, 2211.

Lloyd, G.a.M., B.H. (1981) Woody Plant Medium (WPM)—A Mineral Nutrient Formulation for Microculture of Woody Plant Species. HortScience 16, 453.

Lombi, E., Zhao, F.J., McGrath, S.P., Young, S.D., Sacchi, G.A. (2008) Physiological evidence for a high-affinity cadmium transporter highly expressed in a Thlaspi caerulescens ecotype. New Phytologist 149, 53-60.

Lombi, E., Zhao, F.J., McGrath, S.P., Young, S.D., Sacchi, G.A. (2008) Physiological evidence for a high-affinity cadmium transporter highly expressed in a Thlaspi caerulescens ecotype. New Phytologist 149, 53-60.

Lone, M.I., He, Z.-l., Stoffella, P.J., Yang, X.-e. (2008) Phytoremediation of heavy metal polluted soils and water: progresses and perspectives. Journal of Zhejiang University. Science. B 9, 210-220.

Lou, L.Q., Ye, Z.H., Lin, A.J., Wong, M.H. (2010) Interaction of arsenic and phosphate on their uptake and accumulation in Chinese brake fern. Int J Phytoremediation 12, 487-502.

Lunackova, L., Masarovicova, E., Kral'ova, K., Stresko, V. (2003) Response of fast growing woody plants from family Salicaceae to cadmium treatment. Bull Environ Contam Toxicol 70, 576-585.

Luo, S.L., Chen, L., Chen, J.L., Xiao, X., Xu, T.Y., Wan, Y., Rao, C., Liu, C.B., Liu, Y.T., Lai, C., Zeng, G.M. (2011) Analysis and characterization of cultivable heavy metal-resistant bacterial endophytes isolated from Cd-hyperaccumulator Solanum nigrum L. and their potential use for phytoremediation. Chemosphere 85, 1130-1138.

Ma, J.F., Yamaji, N., Mitani, N., Xu, X.-Y., Su, Y.-H., McGrath, S.P., Zhao, F.-J. (2008) Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. Proceedings of the National Academy of Sciences 105, 9931-9935.

Ma, J.F., Yamaji, N., Mitani, N., Xu, X.-Y., Su, Y.-H., McGrath, S.P., Zhao, F.-J. (2008) Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. Proceedings of the National Academy of Sciences 105, 9931-9935.

Ma, Y., Rajkumar, M., Zhang, C., Freitas, H. (2016) Beneficial role of bacterial endophytes in heavy metal phytoremediation. Journal of Environmental Management 174, 14-25.

Maciaszczyk-Dziubinska, E., Wawrzycka, D., Wysocki, R. (2012) Arsenic and Antimony Transporters in Eukaryotes. International Journal of Molecular Sciences 13, 3527-3548.

Maestri, E., Marmiroli, M., Visioli, G., Marmiroli, N. (2010) Metal tolerance and hyperaccumulation: Costs and trade-offs between traits and environment. Environmental and Experimental Botany 68, 1-13.

Mahar, A., Wang, P., Ali, A., Awasthi, M.K., Lahori, A.H., Wang, Q., Li, R., Zhang, Z. (2016) Challenges and opportunities in the phytoremediation of heavy metals contaminated soils: A review. Ecotoxicology and Environmental Safety 126, 111-121.

Maksymiec, W. (2007) Signaling responses in plants to heavy metal stress. Acta Physiologiae Plantarum 29, 177.

Maksymiec, W. (2011) Effect of jasmonate and some other signaling factors on bean and onion growth during the initial phase of cadmium action.

Mandal, B.K., Suzuki, K.T. (2002) Arsenic round the world: a review. Talanta 58, 201-235.

Mandal, S.M., Chakraborty, D., Dey, S. (2010) Phenolic acids act as signaling molecules in plant-microbe symbioses. Plant signaling & behavior 5, 359-368.

Martínez, J., Saavedra, Á., García-Nieto, P.J., Piñeiro, J.I., Iglesias, C., Taboada, J., Sancho, J., Pastor, J. (2014) Air quality parameters outliers detection using functional data analysis in the Langreo urban area (Northern Spain). Applied Mathematics and Computation 241, 1-10.

Martínez-Sánchez, M.J., Martínez-López, S., García-Lorenzo, M.L., Martínez-Martínez, L.B., Pérez-Sirvent, C. (2011) Evaluation of arsenic in soils and plant uptake using various chemical extraction methods in soils affected by old mining activities. Geoderma 160, 535-541.

Matschullat, J. (2000) Arsenic in the geosphere — a review. Science of The Total Environment 249, 297-312.

Matsumoto, Y., Okada, Y., Min, K.S., Onosaka, S., Tanaka, K. (1990) Amino acids and peptides. XXVII. Synthesis of phytochelatin-related peptides and examination of their heavy metal-binding properties. Chem Pharm Bull (Tokyo) 38, 2364-2368.

Maywald, F., Weigel, H.J. (1997) Biochemistry and molecular biology of heavy metal accumulation in higher plants. Landbauforschung Volkenrode 47, 103-126.

McDonald, M., Mila, I., Scalbert, A. (1996) Precipitation of Metal Ions by Plant Polyphenols: Optimal Conditions and Origin of Precipitation.

McLaughlin, M.J., Zarcinas, B.A., Stevens, D.P., Cook, N. (2000) Soil testing for heavy metals. Communications in Soil Science and Plant Analysis 31, 1661-1700.

McMurdie, P.J., Holmes, S. (2013) phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217.

Meda, A., Lamien, C.E., Romito, M., Millogo, J., Nacoulma, O.G. (2005) Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. Food Chemistry 91, 571-577.

Meers, E., Vandecasteele, B., Ruttens, A., Vangronsveld, J., Tack, F.M.G. (2007) Potential of five willow species (Salix spp.) for phytoextraction of heavy metals. Environmental and Experimental Botany 60, 57-68.

Meharg, A.A., Hartley-Whitaker, J. (2002) Arsenic uptake and metabolism in arsenic resistant and nonresistant plant species. New Phytologist 154, 29-43.

Meharg, A.A., Macnair, M.R. (1994) Relationship between plant phosphorus status and the kinetics of arsenate influx in clones ofdeschampsia cespitosa (L.) beauv. that differ in their tolerance to arsenate. Plant and Soil 162, 99-106.

Mench, M., Lepp, N., Bert, V., Schwitzguébel, J.-P., Gawronski, S.W., Schröder, P., Vangronsveld, J. (2010) Successes and limitations of phytotechnologies at field scale: outcomes, assessment and outlook from COST Action 859. Journal of Soils and Sediments 10, 1039-1070.

Mench, M., Schwitzguébel, J.-P., Schroeder, P., Bert, V., Gawronski, S., Gupta, S. (2009) Assessment of successful experiments and limitations of phytotechnologies: contaminant uptake, detoxification and sequestration, and consequences for food safety. Environmental Science and Pollution Research 16, 876.

Mendoza-Cózatl, D.G., Jobe, T.O., Hauser, F., Schroeder, J.I. (2011) Long-distance transport, vacuolar sequestration, tolerance, and transcriptional responses induced by cadmium and arsenic. Current Opinion in Plant Biology 14, 554-562.

Mergeay, M., Nies, D., Schlegel, H.G., Gerits, J., Charles, P., Van Gijsegem, F. (1985) Alcaligenes eutrophus CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. Journal of bacteriology 162, 328-334.

Mesa, V., Navazas, A., Gonzalez-Gil, R., Gonzalez, A., Weyens, N., Lauga, B., Gallego, J.L., Sanchez, J., Pelaez, A.I. (2017) Use of Endophytic and Rhizosphere Bacteria To Improve Phytoremediation of Arsenic-Contaminated Industrial Soils by Autochthonous Betula celtiberica. Appl Environ Microbiol 83.

Messens, J., Silver, S. (2006) Arsenate reduction: thiol cascade chemistry with convergent evolution. J Mol Biol 362, 1-17.

Meyer, A.J., Hell, R. (2005) Glutathione homeostasis and redox-regulation by sulfhydryl groups. Photosynth Res 86, 435-457.

Mishra, S., Alfeld, M., Sobotka, R., Andresen, E., Falkenberg, G., Kupper, H. (2016) Analysis of sublethal arsenic toxicity to Ceratophyllum demersum: subcellular distribution of arsenic and inhibition of chlorophyll biosynthesis. J Exp Bot 67, 4639-4646.

Mishra, S., Stark, H.J., Kupper, H. (2014) A different sequence of events than previously reported leads to arsenic-induced damage in Ceratophyllum demersum L. Metallomics 6, 444-454.

Mishra, V.K., Upadhyay, A.R., Pandey, S.K., Tripathi, B.D. (2008) Concentrations of heavy metals and aquatic macrophytes of Govind Ballabh Pant Sagar an anthropogenic lake affected by coal mining effluent. Environmental Monitoring and Assessment 141, 49-58.

Mitani-Ueno, N., Yamaji, N., Zhao, F.J., Ma, J.F. (2011) The aromatic/arginine selectivity filter of NIP aquaporins plays a critical role in substrate selectivity for silicon, boron, and arsenic. J Exp Bot 62, 4391-4398.

Mitter, B., Pfaffenbichler, N., Sessitsch, A. (2016) Plant-microbe partnerships in 2020. Microb Biotechnol 9, 635-640.

Mittler, R. (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7, 405-410.

Mleczek, M., Rutkowski, P., Rissmann, I., Kaczmarek, Z., Golinski, P., Szentner, K., Strażyńska, K., Stachowiak, A. (2010) Biomass productivity and phytoremediation potential of Salix alba and Salix viminalis. Biomass and Bioenergy 34, 1410-1418.

Mohan, T.C., Castrillo, G., Navarro, C., Zarco-Fernandez, S., Ramireddy, E., Mateo, C., Zamarreno, A.M., Paz-Ares, J., Munoz, R., Garcia-Mina, J.M., Hernandez, L.E., Schmulling, T., Leyva, A. (2016) Cytokinin Determines Thiol-Mediated Arsenic Tolerance and Accumulation. Plant Physiol 171, 1418-1426.

Mohd, S., Shukla, J., Kushwaha, A.S., Mandrah, K., Shankar, J., Arjaria, N., Saxena, P.N., Narayan, R., Roy, S.K., Kumar, M. (2017) Endophytic Fungi Piriformospora indica Mediated Protection of Host from Arsenic Toxicity. Frontiers in Microbiology 8, 754-754.

Moliterni, E., Rodriguez, L., Fernández, F.J., Villaseñor, J. (2012) Feasibility of Different Bioremediation Strategies for Treatment of Clayey and Silty Soils Recently Polluted with Diesel Hydrocarbons. Water, Air, & Soil Pollution 223, 2473-2482.

Monachese, M., Burton, J.P., Reid, G. (2012) Bioremediation and tolerance of humans to heavy metals through microbial processes: a potential role for probiotics? Appl Environ Microbiol 78, 6397-6404.

Monteiro, C.C., Carvalho, R.F., Gratão, P.L., Carvalho, G., Tezotto, T., Medici, L.O., Peres, L.E.P., Azevedo, R.A. (2011) Biochemical responses of the ethylene-insensitive Never ripe tomato mutant subjected to cadmium and sodium stresses. Environ Exp Bot 71.

Moreno-Jimenez, E., Esteban, E., Fresno, T., de Egea, C.L., Penalosa, J.M. (2010) Hydroponics as a valid tool to assess arsenic availability in mine soils. Chemosphere 79, 513-517.

Morimoto, R.I. (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev 12, 3788-3796.

Morio, M., Schädler, S., Finkel, M. (2013) Applying a multi-criteria genetic algorithm framework for brownfield reuse optimization: Improving redevelopment options based on stakeholder preferences. Journal of Environmental Management 130, 331-346.

Morkunas, I., Woźniak, A., Mai, V.C., Rucińska-Sobkowiak, R., Jeandet, P. (2018) The Role of Heavy Metals in Plant Response to Biotic Stress. Molecules 23, 2320.

Mourato, M., Reis, R., Martins, L., (2012) Characterization of Plant Antioxidative System in Response to Abiotic Stresses: A Focus on Heavy Metal Toxicity.

Mrozik, A., Piotrowska-Seget, Z. (2010) Bioaugmentation as a strategy for cleaning up of soils contaminated with aromatic compounds. Microbiological Research 165, 363-375.

Mukhopadhyay, R., Shi, J., Rosen, B.P. (2000) Purification and characterization of ACR2p, the Saccharomyces cerevisiae arsenate reductase. J Biol Chem 275, 21149-21157.

Murashige, T., Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15, 473-497.

Murota, C., Matsumoto, H., Fujiwara, S., Hiruta, Y., Miyashita, S., Shimoya, M., Kobayashi, I., Hudock, M.O., Togasaki, R.K., Sato, N., Tsuzuki, M. (2012) Arsenic tolerance in a Chlamydomonas photosynthetic mutant is due to reduced arsenic uptake even in light conditions. Planta 236, 1395-1403.

Na, G., Salt, D.E. (2011) The role of sulfur assimilation and sulfur-containing compounds in trace element homeostasis in plants. Environmental and Experimental Botany 72, 18-25.

Na, G., Salt, D.E. (2011) The role of sulfur assimilation and sulfur-containing compounds in trace element homeostasis in plants. Environmental and Experimental Botany 72, 18-25.

Naidu, R., Smith, E., Owens, G., Bhattacharya, P., Nadebaum, P. (2006) Managing arsenic in the environment: from soil to human health. CSIRO Publishing, Collingwood.

Nair, V.D., Panneerselvam, R., Gopi, R. (2012) Studies on methanolic extract of Rauvolfia species from Southern Western Ghats of India – In vitro antioxidant properties, characterisation of nutrients and phytochemicals. Industrial Crops and Products 39, 17-25.

Naka, H., Liu, M., Crosa, J.H. (2013) Two ABC transporter systems participate in siderophore transport in the marine pathogen Vibrio anguillarum 775 (pJM1). FEMS Microbiol Lett 341, 79-86.

Nealson, K.H., Belz, A., McKee, B. (2002) Breathing metals as a way of life: geobiology in action. Antonie Van Leeuwenhoek 81, 215-222.

Neill, S.O., Gould, K.S., Kilmartin, P.A., Mitchell, K.A., Markham, K.R. (2002) Antioxidant activities of red versus green leaves in Elatostema rugosum. Plant Cell Environ 25, 539-547.

Newton, G.L., Buchmeier, N., Fahey, R.C. (2008) Biosynthesis and functions of mycothiol, the unique protective thiol of Actinobacteria. Microbiol Mol Biol Rev 72, 471-494.

Nie, M., Bell, C., Wallenstein, M.D., Pendall, E. (2015) Increased plant productivity and decreased microbial respiratory C loss by plant growth-promoting rhizobacteria under elevated CO(2). Scientific Reports 5, 9212.

Nies, D.H. (2003) Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev 27, 313-339.

Nihorimbere, V., Ongena, M., Smargiassi, M., Thonart, P. (2011) Beneficial effect of the rhizosphere microbial community for plant growth and health.

Noctor, G. (2006) Metabolic signalling in defence and stress: the central roles of soluble redox couples. Plant Cell Environ 29, 409-425.

Noronha, H., Araújo, D., Conde, C., Martins, A.P., Soveral, G., Chaumont, F., Delrot, S., Gerós, H. (2016) The Grapevine Uncharacterized Intrinsic Protein 1 (VvXIP1) Is Regulated by Drought Stress and Transports Glycerol, Hydrogen Peroxide, Heavy Metals but Not Water. PLoS One 11, e0160976-e0160976.

Núñez, O., Fernández-Navarro, P., Martín-Méndez, I., Bel-Lan, A., Locutura, J.F., López-Abente, G. (2016) Arsenic and chromium topsoil levels and cancer mortality in Spain. Environ Sci Pollut Res Int 23, 17664-17675.

Oden, K.L., Gladysheva, T.B., Rosen, B.P. (1994) Arsenate reduction mediated by the plasmid-encoded ArsC protein is coupled to glutathione. Mol Microbiol 12, 301-306.

Ofori, P.A., Mizuno, A., Suzuki, M., Martinoia, E., Reuscher, S., Aoki, K., Shibata, D., Otagaki, S., Matsumoto, S., Shiratake, K. (2018) Genome-wide analysis of ATP binding cassette (ABC) transporters in tomato. PLoS One 13, e0200854.

Oksanen, F.J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P., O'Hara, R.B., Simpson, G., Solymos, P., others (2017) vegan: Community Ecology Package. R package version 2.4-4. http s.

Olaniran, A.O., Balgobind, A., Pillay, B. (2013) Bioavailability of heavy metals in soil: impact on microbial biodegradation of organic compounds and possible improvement strategies. International Journal of Molecular Sciences 14, 10197-10228.

O'Leary, N.A., Wright, M.W., Brister, J.R., Ciufo, S., Haddad, D., McVeigh, R., et al. (2016) Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res 44, D733-745.

Oremland, R.S., Stolz, J.F. (2003) The ecology of arsenic. Science 300, 939-944.

Overbeek, R., Olson, R., Pusch, G.D., Olsen, G.J., Davis, J.J., Disz, T., Edwards, R.A., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A.R., Xia, F., Stevens, R. (2014) The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res 42, D206-D214.

Page, V., Feller, U. (2005) Selective transport of zinc, manganese, nickel, cobalt and cadmium in the root system and transfer to the leaves in young wheat plants. Ann Bot 96, 425-434.

Pajević, S., Borišev, M., Nikolić, N., Arsenov, D.D., Orlović, S., Župunski, M., (2016) Phytoextraction of Heavy Metals by Fast-Growing Trees: A Review, in: Ansari, A.A., Gill, S.S., Gill, R., Lanza, G.R., Newman, L. (Eds.), Phytoremediation: Management of Environmental Contaminants, Volume 3. Springer International Publishing, Cham, pp. 29-64.

Pal, C., Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G. (2015) Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. BMC Genomics 16, 964.

Panda, S.K., Upadhyay, R.K., Nath, S. (2010) Arsenic Stress in Plants. Journal of Agronomy and Crop Science 196, 161-174.

Pande, S., Kost, C. (2017) Bacterial Unculturability and the Formation of Intercellular Metabolic Networks. Trends Microbiol 25, 349-361.

Pandey, C., Augustine, R., Panthri, M., Zia, I., Bisht, N.C., Gupta, M. (2017) Arsenic affects the production of glucosinolate, thiol and phytochemical compounds: A comparison of two Brassica cultivars. Plant Physiology and Biochemistry 111, 144-154.

Pandey, N., Chandrakar, V., Keshavkant, S., (2018) Mitigating Arsenic Toxicity in Plants: Role of Microbiota, in: Hasanuzzaman, M., Nahar, K., Fujita, M. (Eds.), Mechanisms of Arsenic Toxicity and Tolerance in Plants. Springer Singapore, Singapore, pp. 191-218.

Pandey, P., Singh, J., Achary, V.M.M., Reddy, M.K. (2015) Redox homeostasis via gene families of ascorbate-glutathione pathway. Frontiers in Environmental Science 3.

Parisy, V., Poinssot, B., Owsianowski, L., Buchala, A., Glazebrook, J., Mauch, F. (2007) Identification of PAD2 as a gamma-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of Arabidopsis. Plant J 49, 159-172.

Park, J., Song, W.Y., Ko, D., Eom, Y., Hansen, T.H., Schiller, M., Lee, T.G., Martinoia, E., Lee, Y. (2012) The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. Plant J 69, 278-288.

Park, J.H., Han, Y.S., Seong, H.J., Ahn, J.S., Nam, I.H. (2016) Arsenic uptake and speciation in Arabidopsis thaliana under hydroponic conditions. Chemosphere 154, 283-288.

Patra, M., Bhowmik, N., Bandopadhyay, B., Sharma, A. (2004) Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance. Environmental and Experimental Botany 52, 199-223.

Paul, D.S., Hernández-Zavala, A., Walton, F.S., Adair, B.M., Dina, J.D., Matoušek, T., Stýblo, M. (2007) Examination of the Effects of Arsenic on Glucose Homeostasis in Cell Culture and Animal Studies: Development of a Mouse Model for Arsenic-Induced Diabetes. Toxicology and applied pharmacology 222, 305-314.

Paulose, B., Chhikara, S., Coomey, J., Jung, H.I., Vatamaniuk, O., Dhankher, O.P. (2013) A gamma-glutamyl cyclotransferase protects Arabidopsis plants from heavy metal toxicity by recycling glutamate to maintain glutathione homeostasis. Plant Cell 25, 4580-4595.

Payá, A., Rodríguez, N. (2018) Status of local soil contamination in Europe: Revision of the indicator "Progress in the management Contaminated Sites in Europe, EUR 29124 EN, Publications Office of the European Union, Luxembourg.

Pérez-Torres, C.-A., López-Bucio, J., Cruz-Ramírez, A., Ibarra-Laclette, E., Dharmasiri, S., Estelle, M., Herrera-Estrella, L. (2008) Phosphate availability alters lateral root development in Arabidopsis by modulating auxin sensitivity via a mechanism involving the TIR1 auxin receptor. The Plant Cell 20, 3258-3272.

Pickering, I.J., Gumaelius, L., Harris, H.H., Prince, R.C., Hirsch, G., Banks, J.A., Salt, D.E., George, G.N. (2006) Localizing the biochemical transformations of arsenate in a hyperaccumulating fern. Environmental Science & Technology 40, 5010-5014.

Pidlisnyuk, V., Stefanovska, T., Lewis, E.E., Erickson, L.E., Davis, L.C. (2014) Miscanthus as a Productive Biofuel Crop for Phytoremediation. Critical Reviews in Plant Sciences 33, 1-19.

Pilon-Smits, E. (2005) Phytoremediation. Annu Rev Plant Biol 56, 15-39.

Plaza, S., Tearall, K.L., Zhao, F.J., Buchner, P., McGrath, S.P., Hawkesford, M.J. (2007) Expression and functional analysis of metal transporter genes in two contrasting ecotypes of the hyperaccumulator Thlaspi caerulescens. J Exp Bot 58, 1717-1728.

Porra, R.J. (2002) The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynth Res 73, 149-156.

Porter, B.W., Zhu, Y.J., Webb, D.T., Christopher, D.A. (2009) Novel thigmomorphogenetic responses in Carica papaya: touch decreases anthocyanin levels and stimulates petiole cork outgrowths. Ann Bot 103, 847-858.

Powell, K.D., Harper, F.A., Smith, J.A.C. (2002) The Genetic Basis of Metal Hyperaccumulation in Plants AU - Pollard, A. Joseph. Critical Reviews in Plant Sciences 21, 539-566.

Puckett, E.E., Serapiglia, M.J., DeLeon, A.M., Long, S., Minocha, R., Smart, L.B. (2012) Differential expression of genes encoding phosphate transporters contributes to arsenic tolerance and accumulation in shrub willow (Salix spp.). Environmental and Experimental Botany 75, 248-257.

Pulford, I.D., Watson, C. (2003) Phytoremediation of heavy metal-contaminated land by trees—a review. Environ Int 29, 529-540.

Purdy, J.J., Smart, L.B. (2008) Hydroponic Screening of Shrub Willow (Salix Spp.) for Arsenic Tolerance and Uptake. International Journal of Phytoremediation 10, 515-528.

Quaghebeur, M., Rengel, Z. (2003) The distribution of arsenate and arsenite in shoots and roots of Holcus lanatus is influenced by arsenic tolerance and arsenate and phosphate supply. Plant Physiol 132, 1600-1609.

Raab, A., Schat, H., Meharg, A.A., Feldmann, J. (2005) Uptake, translocation and transformation of arsenate and arsenite in sunflower (Helianthus annuus): formation of arsenic–phytochelatin complexes during exposure to high arsenic concentrations. New Phytologist 168, 551-558.

Radzki, W., Gutierrez Mañero, F.J., Algar, E., Lucas García, J.A., García-Villaraco, A., Ramos Solano, B. (2013) Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture. Antonie Van Leeuwenhoek 104, 321-330.

Rahman, A., M. G. Mostofa, M. M. Alam, K. Nahar, M. Hasanuzzaman and M. Fujita (2015). Calcium Mitigates Arsenic Toxicity in Rice Seedlings by Reducing Arsenic Uptake and Modulating the Antioxidant Defense and Glyoxalase Systems and Stress Markers. BioMed research international 2015: 340812-340812.

Rai, A., Tripathi, P., Dwivedi, S., Dubey, S., Shri, M., Kumar, S., Tripathi, P.K., Dave, R., Kumar, A., Singh, R., Adhikari, B., Bag, M., Tripathi, R.D., Trivedi, P.K., Chakrabarty, D., Tuli, R. (2011) Arsenic tolerances in rice (Oryza sativa) have a predominant role in transcriptional regulation of a set of genes including sulphur assimilation pathway and antioxidant system. Chemosphere 82, 986-995.

Ramakrishna, W., Yadav, R., Li, K. (2019) Plant growth promoting bacteria in agriculture: Two sides of a coin. Applied Soil Ecology 138, 10-18.

Raskin, I., Kumar, P.B.A.N., Dushenkov, S., Salt, D.E. (1994) Bioconcentration of heavy metals by plants. Current Opinion in Biotechnology 5, 285-290.

Rauret, G., F. López-Sánchez, J., Sahuquillo, A., Rubio, R., Davidson, C., Ure, A., Quevauviller, P. (1999) Improvement of the BCR three step sequential extraction procedure

prior to the certification of new sediment and soil reference materials. Journal of Environmental Monitoring 1, 57-61.

Rea, P.A., Vatamaniuk, O.K., Rigden, D.J. (2004) Weeds, Worms, and More. Papain's Long-Lost Cousin, Phytochelatin Synthase. Plant Physiol 136, 2463-2474.

Reichard, J.F., Puga, A. (2010) Effects of arsenic exposure on DNA methylation and epigenetic gene regulation. Epigenomics 2, 87-104.

Reinhold-Hurek, B., Hurek, T. (1998) Life in grasses: diazotrophic endophytes. Trends Microbiol 6, 139-144.

Remans, T., Keunen, E., Bex, G.J., Smeets, K., Vangronsveld, J., Cuypers, A. (2014) Reliable gene expression analysis by reverse transcription-quantitative PCR: reporting and minimizing the uncertainty in data accuracy. Plant Cell 26, 3829-3837.

Remans, T., Smeets, K., Opdenakker, K., Mathijsen, D., Vangronsveld, J., Cuypers, A. (2008) Normalisation of real-time RT-PCR gene expression measurements in Arabidopsis thalianaexposed to increased metal concentrations. Planta 227.

Remans, T., Thijs, S., Truyens, S., Weyens, N., Schellingen, K., Keunen, E., Gielen, H., Cuypers, A., Vangronsveld, J. (2012) Understanding the development of roots exposed to contaminants and the potential of plant-associated bacteria for optimization of growth. Ann Bot 110, 239-252.

Richau, K.H., Kozhevnikova, A.D., Seregin, I.V., Vooijs, R., Koevoets, P.L., Smith, J.A., Ivanov, V.B., Schat, H. (2009) Chelation by histidine inhibits the vacuolar sequestration of nickel in roots of the hyperaccumulator Thlaspi caerulescens. New Phytol 183, 106-116.

Rinklebe, J., Shaheen, S.M. (2014) Assessing the Mobilization of Cadmium, Lead, and Nickel Using a Seven-Step Sequential Extraction Technique in Contaminated Floodplain Soil Profiles Along the Central Elbe River, Germany. Water, Air, & Soil Pollution 225, 2039.

Rockwood, D.L., Naidu, C.V., Carter, D.R., Rahmani, M., Spriggs, T.A., Lin, C., Alker, G.R., Isebrands, J.G., Segrest, S.A. (2004) Short-rotation woody crops and phytoremediation: Opportunities for agroforestry? Agroforestry Systems 61, 51-63.

Rosen, B.P. (2002) Biochemistry of arsenic detoxification. FEBS Lett 529, 86-92.

Rosselli, W., Keller, C., Boschi, K. (2003) Phytoextraction capacity of trees growing on a metal contaminated soil. Plant and Soil 256, 265-272.

Rousk, J., Brookes, P.C., Bååth, E. (2009) Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Appl Environ Microbiol 75, 1589-1596.

Rugova, A., Puschenreiter, M., Santner, J., Fischer, L., Neubauer, S., Koellensperger, G., Hann, S. (2014) Speciation analysis of orthophosphate and myo-inositol hexakisphosphate in soil- and plant-related samples by high-performance ion chromatography combined with inductively coupled plasma mass spectrometry. J Sep Sci 37, 1711-1719.

Ruttens, A., Boulet, J., Weyens, N., Smeets, K., Adriaensen, K., Meers, E., Van Slycken, S., Tack, F., Meiresonne, L., Thewys, T., Witters, N., Carleer, R., Dupae, J., Vangronsveld, J. (2011) Short Rotation Coppice Culture of Willows and Poplars as Energy Crops on Metal Contaminated Agricultural Soils. International Journal of Phytoremediation 13, 194-207.

Salt, D.E., Baxter, I., Lahner, B. (2008) Ionomics and the study of the plant ionome. Annu Rev Plant Biol 59, 709-733.

Salt, D.E., Smith, R.D., Raskin, I. (1998) Phytoremediarion. Annual Review of Plant Physiology and Plant Molecular Biology 49, 643-668.

Sánchez-Bermejo, E., Castrillo, G., del Llano, B., Navarro, C., Zarco-Fernández, S., Martinez-Herrera, D.J., Leo-del Puerto, Y., Muñoz, R., Cámara, C., Paz-Ares, J., Alonso-Blanco,

C., Leyva, A. (2014) Natural variation in arsenate tolerance identifies an arsenate reductase in Arabidopsis thaliana. Nature Communications 5, 4617.

Sandhya, V., Skz, A., Grover, M., Reddy, G., Venkateswarlu, B. (2009) Alleviation of drought stress effects in sunflower seedlings by the exopolysaccharides producing Pseudomonas putida strain GAP-P45.

Sarkar, A., Kazy, S.K., Sar, P. (2013) Characterization of arsenic resistant bacteria from arsenic rich groundwater of West Bengal, India. Ecotoxicology 22, 363-376.

Sarwar, N., Imran, M., Shaheen, M.R., Ishaque, W., Kamran, M.A., Matloob, A., Rehim, A., Hussain, S. (2017) Phytoremediation strategies for soils contaminated with heavy metals: Modifications and future perspectives. Chemosphere 171, 710-721.

Sasse, J., Martinoia, E., Northen, T. (2018) Feed Your Friends: Do Plant Exudates Shape the Root Microbiome? Trends Plant Sci 23, 25-41.

Schat, H., Llugany, M., Vooijs, R., Hartley-Whitaker, J., Bleeker, P.M. (2002) The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. J Exp Bot 53, 2381-2392.

Schellingen, K., Van Der Straeten, D., Remans, T., Vangronsveld, J., Keunen, E., Cuypers, A. (2015) Ethylene signalling is mediating the early cadmium-induced oxidative challenge in Arabidopsis thaliana. Plant Sci 239, 137-146.

Schlegel, H.G., Kaltwasser, H., Gottschalk, G. (1961) [A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies]. Arch Mikrobiol 38, 209-222.

Schlegel, H.G., Kaltwasser, H., Gottschalk, G. (1961) [A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies]. Arch Mikrobiol 38, 209-222.

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C(T) method." Nat Protoc 3(6): 1101-1108.

Schmöger, M.E.V., Oven, M., Grill, E. (2000) Detoxification of Arsenic by Phytochelatins in Plants. Plant Physiol 122, 793-802.

Schnaubelt, D., Schulz, P., Hannah, M., Yocgo, R., Foyer, C. (2013) A phenomics approach to the analysis of the influence of glutathione on leaf area and abiotic stress tolerance in Arabidopsis thaliana. Front Plant Sci 4.

Schutzendubel, A., Polle, A. (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J Exp Bot 53, 1351-1365.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C. (2011) Metagenomic biomarker discovery and explanation. Genome Biol 12, R60.

Sessitsch, A., Hardoim, P., Doring, J., Weilharter, A., Krause, A., Woyke, T., Mitter, B., Hauberg-Lotte, L., Friedrich, F., Rahalkar, M., Hurek, T., Sarkar, A., Bodrossy, L., van Overbeek, L., Brar, D., van Elsas, J.D., Reinhold-Hurek, B. (2012) Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. Mol Plant Microbe Interact 25, 28-36.

Sessitsch, A., Kuffner, M., Kidd, P., Vangronsveld, J., Wenzel, W.W., Fallmann, K., Puschenreiter, M. (2013) The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. Soil Biology and Biochemistry 60, 182-194.

Shaheen, S.M., Rinklebe, J. (2015) Impact of emerging and low cost alternative amendments on the (im)mobilization and phytoavailability of Cd and Pb in a contaminated floodplain soil. Ecological Engineering 74, 319-326.

Shaibur, M. R., N. Kitajima, R. Sugawara, T. Kondo, S. M. Imamul Huq and S. Kawai (2008). Physiological and Mineralogical Properties of Arsenic-Induced Chlorosis in Barley Seedlings Grown Hydroponically. Journal of Plant Nutrition 31(2): 333-353.

Shaibur, M.R., Adjadeh, T.A., Kawai, S. (2013) Effect of phosphorus on the concentrations of arsenic, iron and some other elements in barley grown hydroponically. Journal of soil science and plant nutrition 13, 79-85.

Shaibur, M.R., Kitajima, N., Sugawara, R., Kondo, T., Imamul Huq, S.M., Kawai, S. (2008) Physiological and Mineralogical Properties of Arsenic-Induced Chlorosis in Barley Seedlings Grown Hydroponically. Journal of Plant Nutrition 31, 333-353.

Sharma, A., Patni, B., Shankhdhar, D., Shankhdhar, S.C. (2013a) Zinc - an indispensable micronutrient. Physiology and molecular biology of plants : an international journal of functional plant biology 19, 11-20.

Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M. (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of botany 2012.

Sharma, R., Bhardwaj, R., Handa, N., Gautam, V., Kohli, S.K., Bali, S., Kaur, P., Thukral, A.K., Arora, S., Ohri, P., Vig, A.P., (2016) Chapter 10 - Responses of Phytochelatins and Metallothioneins in Alleviation of Heavy Metal Stress in Plants: An Overview, in: Ahmad, P. (Ed.), Plant Metal Interaction. Elsevier, pp. 263-283.

Sharma, R., R. Bhardwaj, N. Handa, V. Gautam, S. K. Kohli, S. Bali, P. Kaur, A. K. Thukral, S. Arora, P. Ohri and A. P. Vig (2016). Responses of Phytochelatins and Metallothioneins in Alleviation of Heavy Metal Stress in Plants: An Overview. Plant Metal Interaction. P. Ahmad, Elsevier: 263-283.

Sharma, S.B., Sayyed, R.Z., Trivedi, M.H., Gobi, T.A. (2013b) Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. SpringerPlus 2, 587-587.

Shelmerdine, P.A., Black, C.R., McGrath, S.P., Young, S.D. (2009) Modelling phytoremediation by the hyperaccumulating fern, Pteris vittata, of soils historically contaminated with arsenic. Environmental Pollution 157, 1589-1596.

Shi, G., Liu, C., Cai, Q., Liu, Q., Hou, C. (2010) Cadmium accumulation and tolerance of two safflower cultivars in relation to photosynthesis and antioxidative enzymes. Bull Environ Contam Toxicol 85, 256-263.

Shin, H., Shin, H.S., Dewbre, G.R., Harrison, M.J. (2004) Phosphate transport in Arabidopsis: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. Plant J 39, 629-642.

Sierra, C., Gallego, J.R., Afif, E., Menéndez-Aguado, J.M., González-Coto, F. (2010) Analysis of soil washing effectiveness to remediate a brownfield polluted with pyrite ashes. Journal of Hazardous Materials 180, 602-608.

Sierra, M.J., Millán, R., Esteban, E., Cardona, A.I., Schmid, T. (2008) Evaluation of mercury uptake and distribution in Vicia sativa L. applying two different study scales: Greenhouse conditions and lysimeter experiments. Journal of Geochemical Exploration 96, 203-209.

Silver, S., Phung, L.T. (2005) Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. Appl Environ Microbiol 71, 599-608.

Singh, A.P., Goel, R.K., Kaur, T. (2011) Mechanisms Pertaining to Arsenic Toxicity. Toxicology International 18, 87-93.

Singh, H.P., Batish, D.R., Kohli, R.K., Arora, K. (2007) Arsenic-induced root growth inhibition in mung bean (Phaseolus aureus Roxb.) is due to oxidative stress resulting from enhanced lipid peroxidation. Plant Growth Regulation 53, 65-73.

Singh, N., Ma, L.Q., Srivastava, M., Rathinasabapathi, B. (2006) Metabolic adaptations to arsenic-induced oxidative stress in Pterisvittata L and Pterisensiformis L. Plant Science 170, 274-282.

Singh, N., Marwa, N., Mishra, S.K., Mishra, J., Verma, P.C., Rathaur, S., Singh, N. (2016) Brevundimonas diminuta mediated alleviation of arsenic toxicity and plant growth promotion in Oryza sativa L. Ecotoxicol Environ Saf 125, 25-34.

Skvortsov, A.K. (1999) Willows of Russia and adjacent countries: taxonomical and geographical revision. University of Joensuu, Joensuu, pp.307.

Smith, A. H., A. Ercumen, Y. Yuan and C. M. Steinmaus (2009). "Increased lung cancer risks are similar whether arsenic is ingested or inhaled." Journal of exposure science & environmental epidemiology 19(4): 343-348.

Sneller, F. E. C., van Heerwaarden, L. M., Kraaijeveld-Smit, F. J. L., ten Bookum, W. M., Koevoets, P. L. M., Schat, H., & Verkley, J. A. C. (1999). Toxicity of arsenate in Silene vulgaris, accumulation and degradation of arsenate-induced phytochelatins. *New Phytologist*, (144), 223-232.

Socha, A., Guerinot, M.L. (2014) Mn-euvering manganese: the role of transporter gene family members in manganese uptake and mobilization in plants. Front Plant Sci 5.

Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., Arrieta, J.M., Herndl, G.J. (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". Proceedings of the National Academy of Sciences 103, 12115-12120.

Sok, J., Calfon, M., Lu, J., Lichtlen, P., Clark, S.G., Ron, D. (2001) Arsenite-inducible RNA-associated protein (AIRAP) protects cells from arsenite toxicity. Cell Stress Chaperones 6, 6-15.

Song, W.Y., Park, J., Mendoza-Cozatl, D.G., Suter-Grotemeyer, M., Shim, D., Hortensteiner, S., Geisler, M., Weder, B., Rea, P.A., Rentsch, D., Schroeder, J.I., Lee, Y., Martinoia, E. (2010) Arsenic tolerance in Arabidopsis is mediated by two ABCC-type phytochelatin transporters. Proc Natl Acad Sci U S A 107, 21187-21192.

Song, W.Y., Park, J., Mendoza-Cozatl, D.G., Suter-Grotemeyer, M., Shim, D., Hortensteiner, S., Geisler, M., Weder, B., Rea, P.A., Rentsch, D., Schroeder, J.I., Lee, Y., Martinoia, E. (2010) Arsenic tolerance in Arabidopsis is mediated by two ABCC-type phytochelatin transporters. Proc Natl Acad Sci U S A 107, 21187-21192.

Spratlen, M.J., Gamble, M.V., Grau-Perez, M., Kuo, C.-C., Best, L.G., Yracheta, J., Francesconi, K., Goessler, W., Mossavar-Rahmani, Y., Hall, M., Umans, J.G., Fretts, A., Navas-Acien, A. (2017) Arsenic metabolism and one-carbon metabolism at low-moderate arsenic exposure: Evidence from the Strong Heart Study. Food and Chemical Toxicology 105, 387-397.

Spratlen, M.J., Gamble, M.V., Grau-Perez, M., Kuo, C.-C., Best, L.G., Yracheta, J., Francesconi, K., Goessler, W., Mossavar-Rahmani, Y., Hall, M., Umans, J.G., Fretts, A., Navas-Acien, A. (2017) Arsenic metabolism and one-carbon metabolism at low-moderate arsenic exposure: Evidence from the Strong Heart Study. Food and Chemical Toxicology 105, 387-397.

Srivastava, M., Ma, L.Q., Singh, N., Singh, S. (2005) Antioxidant responses of hyper-accumulator and sensitive fern species to arsenic. J Exp Bot 56, 1335-1342.

Srivastava, S., Shrivastava, M. (2017) Zinc supplementation imparts tolerance to arsenite stress in Hydrilla verticillata (L.f.) Royle. Int J Phytoremediation 19, 353-359.

Srivastava, S., Sinha, P., Sharma, Y.K. (2017) Status of photosynthetic pigments, lipid peroxidation and anti-oxidative enzymes in Vigna mungo in presence of arsenic. Journal of Plant Nutrition 40, 298-306.

Srivastava, S., Srivastava, A.K., Sablok, G., Deshpande, T.U., Suprasanna, P. (2015) Transcriptomics profiling of Indian mustard (Brassica juncea) under arsenate stress identifies key candidate genes and regulatory pathways. Front Plant Sci 6, 646.

Stanhill, A., Haynes, C.M., Zhang, Y., Min, G., Steele, M.C., Kalinina, J., Martinez, E., Pickart, C.M., Kong, X.-P., Ron, D. (2006) An Arsenite-Inducible 19S Regulatory Particle-Associated Protein Adapts Proteasomes to Proteotoxicity. Molecular Cell 23, 875-885.

Stein, M., Dittgen, J., Sanchez-Rodriguez, C., Hou, B.H., Molina, A., Schulze-Lefert, P., Lipka, V., Somerville, S. (2006) Arabidopsis PEN3/PDR8, an ATP binding cassette

transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18, 731-746.

Straif, K., Benbrahim-Tallaa, L., Baan, R., Grosse, Y., Secretan, B., El Ghissassi, F., Bouvard, V., Guha, N., Freeman, C., Galichet, L. (2009) A review of human carcinogens—part C: metals, arsenic, dusts, and fibres. The lancet oncology 10, 453-454.

Sturz, A.V., Christie, B.R., Nowak, J. (2000) Bacterial Endophytes: Potential Role in Developing Sustainable Systems of Crop Production. Critical Reviews in Plant Sciences 19, 1-30.

Suleimanova, A.D., Beinhauer, A., Valeeva, L.R., Chastukhina, I.B., Balaban, N.P., Shakirov, E.V., Greiner, R., Sharipova, M.R. (2015) Novel Glucose-1-Phosphatase with High Phytase Activity and Unusual Metal Ion Activation from Soil Bacterium Pantoea sp. Strain 3.5.1. Appl Environ Microbiol 81, 6790-6799.

Sun, B., Ricardo-da-Silva, J.M., Spranger, I. (1998) Critical Factors of Vanillin Assay for Catechins and Proanthocyanidins. Journal of Agricultural and Food Chemistry 46, 4267-4274.

Sung, D.Y., Kim, T.H., Komives, E.A., Mendoza-Cozatl, D.G., Schroeder, J.I. (2009) ARS5 is a component of the 26S proteasome complex, and negatively regulates thiol biosynthesis and arsenic tolerance in Arabidopsis. Plant J 59, 802-813.

Sungur, A., Soylak, M., Ozcan, H. (2014) Investigation of heavy metal mobility and availability by the BCR sequential extraction procedure: relationship between soil properties and heavy metals availability. Chemical Speciation & Bioavailability 26, 219-230.

Sylvain, B., Mikael, M.-H., Florie, M., Emmanuel, J., Marilyne, S., Sylvain, B., Domenico, M. (2016) Phytostabilization of As, Sb and Pb by two willow species (S. viminalis and S. purpurea) on former mine technosols. CATENA 136, 44-52.

Taiz, L. Zeiger, E., Moller, I.M. and Murphy, A. (2015) Plant Physiology and Development. 6th Edition, Sinauer Associates, Sunderland, CT.

Takahashi, H., Kopriva, S., Giordano, M., Saito, K., Hell, R. (2011) Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annu Rev Plant Biol 62, 157-184.

Takahashi, Y., Minamikawa, R., Hattori, K.H., Kurishima, K., Kihou, N., Yuita, K. (2004) Arsenic behavior in paddy fields during the cycle of flooded and non-flooded periods. Environmental Science & Technology 38, 1038-1044.

Talukdar, D., (2017) Balancing Roles of Reactive Oxygen Species in Plants' Response to Metalloid Exposure: Boon Or Bane - Revisiting the Role of ROS, pp. 51-73.

Tamás, M.J., Sharma, S.K., Ibstedt, S., Jacobson, T., Christen, P. (2014) Heavy metals and metalloids as a cause for protein misfolding and aggregation. Biomolecules 4, 252-267.

Tangahu, B.V., Abdullah, S., Rozaimah, S., Basri, H., Idris, M., Anuar, N., Mukhlisin, M. (2011) A review on heavy metals (As, Pb, and Hg) uptake by plants through phytoremediation. International Journal of Chemical Engineering, vol 2011, pp.31.

Tanghe, A., Van Dijck, P., Thevelein, J.M. (2006) Why do microorganisms have aquaporins? Trends Microbiol 14, 78-85.

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J. (2012) Heavy Metals Toxicity and the Environment. EXS 101, 133-164.

Thao, N.P., Khan, M.I.R., Thu, N.B.A., Hoang, X.L.T., Asgher, M., Khan, N.A., Tran, L.-S.P. (2015) Role of Ethylene and Its Cross Talk with Other Signaling Molecules in Plant Responses to Heavy Metal Stress. Plant Physiol 169, 73-84.

Thewys, T., Witters, N., Meers, E., Vangronsveld, J. (2010) Economic Viability of Phytoremediation of a Cadmium Contaminated Agricultural Area Using Energy Maize. Part II: Economics of Anaerobic Digestion of Metal Contaminated Maize in Belgium. International Journal of Phytoremediation 12, 663-679.

Thijs, S., Langill, T., Vangronsveld, J., (2017) Chapter Two - The Bacterial and Fungal Microbiota of Hyperaccumulator Plants: Small Organisms, Large Influence, in: Cuypers, A., Vangronsveld, J. (Eds.), Advances in Botanical Research. Academic Press, pp. 43-86.

Thijs, S., Sillen, W., Rineau, F., Weyens, N., Vangronsveld, J. (2016) Towards an Enhanced Understanding of Plant-Microbiome Interactions to Improve Phytoremediation: Engineering the Metaorganism. Frontiers in Microbiology 7, 341-341.

Thijs, S., Witters, N., Janssen, J., Ruttens, A., Weyens, N., Herzig, R., Mench, M., Van Slycken, S., Meers, E., Meiresonne, L., Vangronsveld, J. (2018) Tobacco, Sunflower and High Biomass SRC Clones Show Potential for Trace Metal Phytoextraction on a Moderately Contaminated Field Site in Belgium. Front Plant Sci 9.

Thompson, J.D., Higgins, D.G., Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22, 4673-4680.

Thongnok, S., Siripornadulsil, W., Siripornadulsil, S. (2018) Mitigation of arsenic toxicity and accumulation in hydroponically grown rice seedlings by co-inoculation with arsenite-oxidizing and cadmium-tolerant bacteria. Ecotoxicology and Environmental Safety 162, 591-602.

Tlustoš, P., Goessler, W., Száková, J.i., Balík, J.i. (2002) Arsenic compounds in leaves and roots of radish grown in soil treated by arsenite, arsenate and dimethylarsinic acid. Applied Organometallic Chemistry 16, 216-220.

Tlustoš, P., Száková, J.i., Vysloužilová, M., Pavlíková, D., Weger, J., Javorská, H. (2007) Variation in the uptake of Arsenic, Cadmium, Lead, and Zinc by different species of willows Salix spp. grown in contaminated soils. Central European Journal of Biology 2, 254-275.

Tokunaga, S., Hakuta, T. (2002) Acid washing and stabilization of an artificial arsenic-contaminated soil. Chemosphere 46, 31-38.

Tóth, G., Hermann, T., Da Silva, M.R., Montanarella, L. (2016a) Heavy metals in agricultural soils of the European Union with implications for food safety. Environ Int 88, 299-309.

Tóth, G., Hermann, T., Szatmári, G., Pásztor, L. (2016b) Maps of heavy metals in the soils of the European Union and proposed priority areas for detailed assessment. Science of The Total Environment 565, 1054-1062.

Tripathi, P., Dwivedi, S., Mishra, A., Kumar, A., Dave, R., Srivastava, S., Shukla, M.K., Srivastava, P.K., Chakrabarty, D., Trivedi, P.K., Tripathi, R.D. (2012) Arsenic accumulation in native plants of West Bengal, India: prospects for phytoremediation but concerns with the use of medicinal plants. Environmental Monitoring and Assessment 184, 2617-2631.

Tsai, S.L., Singh, S., Chen, W. (2009) Arsenic metabolism by microbes in nature and the impact on arsenic remediation. Curr Opin Biotechnol 20, 659-667.

Tsuji, N., Hirayanagi, N., Okada, M., Miyasaka, H., Hirata, K., Zenk, M.H., Miyamoto, K. (2002) Enhancement of tolerance to heavy metals and oxidative stress in Dunaliella tertiolecta by Zn-induced phytochelatin synthesis. Biochem Biophys Res Commun 293, 653-659.

Tu, C., Ma, L.Q. (2003) Effects of arsenate and phosphate on their accumulation by an arsenic-hyperaccumulator Pteris vittata L. Plant and Soil 249, 373-382.

Tuli, R., Chakrabarty, D., Trivedi, P., Deo Tripathi, R. (2010) Recent advances in arsenic accumulation and metabolism in rice.

Turner, T.R., Ramakrishnan, K., Walshaw, J., Heavens, D., Alston, M., Swarbreck, D., Osbourn, A., Grant, A., Poole, P.S. (2013) Comparative metatranscriptomics reveals kingdom level changes in the rhizosphere microbiome of plants. The Isme Journal 7, 2248.

U.S. EPA. 1996. Technology Screening Guide for Radioactively Contaminated Sites, EPA 402/R-96 /017. Office of Radiation and Office of Solid Waste and Emergency Response, 203 pp.

Unterbrunner, R., Puschenreiter, M., Sommer, P., Wieshammer, G., Tlustoš, P., Zupan, M., Wenzel, W.W. (2007) Heavy metal accumulation in trees growing on contaminated sites in Central Europe. Environmental Pollution 148, 107-114.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G. (2012) Primer3--new capabilities and interfaces. Nucleic Acids Res 40, e115.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G. (2012) Primer3--new capabilities and interfaces. Nucleic Acids Res 40, e115.

Upadhyay, M.K., Yadav, P., Shukla, A., Srivastava, S. (2018) Utilizing the Potential of Microorganisms for Managing Arsenic Contamination: A Feasible and Sustainable Approach. Frontiers in Environmental Science 6.

Van de Poel, B., Van Der Straeten, D. (2014) 1-aminocyclopropane-1-carboxylic acid (ACC) in plants: more than just the precursor of ethylene! Front Plant Sci 5, 640.

van der Heijden, M.G., Bardgett, R.D., van Straalen, N.M. (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol Lett 11, 296-310.

Van Slycken, S., Witters, N., Meiresonne, L., Meers, E., Ruttens, A., Van Peteghem, P., Weyens, N., Tack, F.M., Vangronsveld, J. (2013) Field evaluation of willow under short rotation coppice for phytomanagement of metal-polluted agricultural soils. Int J Phytoremediation 15, 677-689.

Vance, C.P., Uhde-Stone, C., Allan, D.L. (2003) Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New Phytologist 157, 423-447.

Vanderauwera, S., Zimmermann, P., Rombauts, S., Vandenabeele, S., Langebartels, C., Gruissem, W., Inze, D., Van Breusegem, F. (2005) Genome-wide analysis of hydrogen peroxide-regulated gene expression in Arabidopsis reveals a high light-induced transcriptional cluster involved in anthocyanin biosynthesis. Plant Physiol 139, 806-821.

Vangronsveld, J., Herzig, R., Weyens, N., Boulet, J., Adriaensen, K., Ruttens, A., Thewys, T., Vassilev, A., Meers, E., Nehnevajova, E., van der Lelie, D., Mench, M. (2009) Phytoremediation of contaminated soils and groundwater: lessons from the field. Environmental Science and Pollution Research 16, 765-794.

Verbruggen, N., Hermans, C., Schat, H. (2009) Molecular mechanisms of metal hyperaccumulation in plants. New Phytol 181, 759-776.

Verkleij, J.A.C., Golan-Goldhirsh, A., Antosiewisz, D.M., Schwitzguébel, J.-P., Schröder, P. (2009) Dualities in plant tolerance to pollutants and their uptake and translocation to the upper plant parts. Environmental and Experimental Botany 67, 10-22.

Viehweger, K. (2014) How plants cope with heavy metals. Botanical studies 55, 35-35.

Vogel, T.M. (1996) Bioaugmentation as a soil bioremediation approach. Current Opinion in Biotechnology 7, 311-316.

Vurro, E., Ruotolo, R., Ottonello, S., Elviri, L., Maffini, M., Falasca, G., Zanella, L., Altamura, M.M., Sanità di Toppi, L. (2011) Phytochelatins govern zinc/copper homeostasis and cadmium detoxification in Cuscuta campestris parasitizing Daucus carota. Environmental and Experimental Botany 72, 26-33.

Vysloužilová, M., Tlustoš, P., Száková, J. (2003) Cadmium and Zinc Phytoextraction Potential of Seven Clones of Salix spp. Planted on Heavy Metal Contaminated Soils.

Walker, J.J., Pace, N.R. (2007) Phylogenetic composition of Rocky Mountain endolithic microbial ecosystems. Appl Environ Microbiol 73, 3497-3504.

Wang, J., Zhao, F.J., Meharg, A.A., Raab, A., Feldmann, J., McGrath, S.P. (2002) Mechanisms of arsenic hyperaccumulation in Pteris vittata. Uptake kinetics, interactions with phosphate, and arsenic speciation. Plant Physiol 130, 1552-1561.

Wang, L., Zhuang, X., Zhuang, G., Jing, C. (2016) Arsenic resistance strategy in Pantoea sp. IMH: Organization, function and evolution of ars genes. Scientific Reports 6, 39195-39195.

Wang, X., Mu, X., Zhang, J., Huang, Q., Alamdar, A., Tian, M., Liu, L., Shen, H. (2015) Serum metabolomics reveals that arsenic exposure disrupted lipid and amino acid metabolism in rats: a step forward in understanding chronic arsenic toxicity. Metallomics 7, 544-552.

Watt, M., Silk, W.K., Passioura, J.B. (2006) Rates of root and organism growth, soil conditions, and temporal and spatial development of the rhizosphere. Ann Bot 97, 839-855.

Wenzel, W.W., Bunkowski, M., Puschenreiter, M., Horak, O. (2003) Rhizosphere characteristics of indigenously growing nickel hyperaccumulator and excluder plants on serpentine soil. Environmental Pollution 123, 131-138.

Weston, L.A., Mathesius, U. (2013) Flavonoids: their structure, biosynthesis and role in the rhizosphere, including allelopathy. J Chem Ecol 39, 283-297.

Weyens, N., Beckers, B., Schellingen, K., Ceulemans, R., Croes, S., Janssen, J., Haenen, S., Witters, N., Vangronsveld, J. (2013) Plant-associated bacteria and their role in the success or failure of metal phytoextraction projects: first observations of a field-related experiment. Microb Biotechnol 6, 288-299.

Weyens, N., Taghavi, S., Barac, T., van der Lelie, D., Boulet, J., Artois, T., Carleer, R., Vangronsveld, J. (2009a) Bacteria associated with oak and ash on a TCE-contaminated site: characterization of isolates with potential to avoid evapotranspiration of TCE. Environ Sci Pollut Res Int 16, 830-843.

Weyens, N., van der Lelie, D., Artois, T., Smeets, K., Taghavi, S., Newman, L., Carleer, R., Vangronsveld, J. (2009b) Bioaugmentation with Engineered Endophytic Bacteria Improves Contaminant Fate in Phytoremediation. Environmental Science & Technology 43, 9413-9418.

Weyens, N., van der Lelie, D., Taghavi, S., Vangronsveld, J. (2009c) Phytoremediation: plant-endophyte partnerships take the challenge. Curr Opin Biotechnol 20, 248-254.

White, P.J., Brown, P.H. (2010) Plant nutrition for sustainable development and global health. Ann Bot 105, 1073-1080.

WHO (2003) Arsenic in drinking-water: background document for development of WHO guidelines for drinking-water quality. World Health Organization.

Wickham, H. (2009) Ggplot2: Elegant Graphics for Data Analysis.

Williams, L., Salt, D.E. (2009) The plant ionome coming into focus. Current Opinion in Plant Biology 12, 247-249.

Winkel-Shirley, B. (2002) Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol 5, 218-223.

Witters, N., Mendelsohn, R., Passel, S., Van Slycken, S., Weyens, N., Schreurs, E., Meers, E., Tack, F., Vanheusden, B., Vangronsveld, J. (2012a) Phytoremediation, a sustainable remediation technology? II: Economic assessment of CO2 abatement through the use of phytoremediation crops for renewable energy production.

Witters, N., Mendelsohn, R.O., Van Slycken, S., Weyens, N., Schreurs, E., Meers, E., Tack, F., Carleer, R., Vangronsveld, J. (2012b) Phytoremediation, a sustainable remediation technology? Conclusions from a case study. I: Energy production and carbon dioxide abatement. Biomass and Bioenergy 39, 454-469.

Witters, N., Slycken, S., Ruttens, A., Adriaensen, K., Meers, E., Meiresonne, L., Tack, F.M.G., Thewys, T., Laes, E., Vangronsveld, J. (2009) Short-rotation coppice of willow for phytoremediation of a metal-contaminated agricultural area: a sustainability assessment. BioEnergy Res 2.

Wozniak, A., Drzewiecka, K., Kesy, J., Marczak, L., Narozna, D., Grobela, M., Motala, R., Bocianowski, J., Morkunas, I. (2017) The Influence of Lead on Generation of Signalling Molecules and Accumulation of Flavonoids in Pea Seedlings in Response to Pea Aphid Infestation. Molecules 22.

Wu, Q., Du, J., Zhuang, G., Jing, C. (2013) Bacillus sp. SXB and Pantoea sp. IMH, aerobic As(V)-reducing bacteria isolated from arsenic-contaminated soil. J Appl Microbiol 114, 713-721.

Wu, T.-H., Liao, M.-H., Kuo, W.-Y., Huang, C.-H., Hsieh, H.-L., Jinn, T.-L. (2011) Characterization of copper/zinc and manganese superoxide dismutase in green bamboo (Bambusa oldhamii): Cloning, expression and regulation. Plant Physiology and Biochemistry 49, 195-200.

Wu, Z., Liu, Q., Li, Z., Cheng, W., Sun, J., Guo, Z., Li, Y., Zhou, J., Meng, D., Li, H., Lei, P., Yin, H. (2018) Environmental factors shaping the diversity of bacterial communities that promote rice production. BMC Microbiology 18, 51.

Wu, Z., Ren, H., McGrath, S.P., Wu, P., Zhao, F.-J. (2011) Investigating the Contribution of the Phosphate Transport Pathway to Arsenic Accumulation in Rice. Plant Physiol 157, 498-508.

Wysocki, R., Clemens, S., Augustyniak, D., Golik, P., Maciaszczyk, E., Tamas, M.J., Dziadkowiec, D. (2003) Metalloid tolerance based on phytochelatins is not functionally equivalent to the arsenite transporter Acr3p. Biochem Biophys Res Commun 304, 293-300.

Xu, W., Dai, W., Yan, H., Li, S., Shen, H., Chen, Y., Xu, H., Sun, Y., He, Z., Ma, M. (2015) Arabidopsis NIP3;1 Plays an Important Role in Arsenic Uptake and Root-to-Shoot Translocation under Arsenite Stress Conditions. Molecular Plant 8, 722-733.

Xu, X.Y., McGrath, S.P., Zhao, F.J. (2007) Rapid reduction of arsenate in the medium mediated by plant roots. New Phytologist 176, 590-599.

Yadav, K., Gupta, N., Kumar, A., Reece, L.M., Singh, N., Rezania, S., Ahmad Khan, S. (2018) Mechanistic understanding and holistic approach of phytoremediation: A review on application and future prospects. Ecological Engineering 120, 274-298.

Yadav, S.K. (2010) Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. South African Journal of Botany 76, 167-179.

Yan, X.L., Lin, L.Y., Liao, X.Y., Zhang, W.B. (2012) Arsenic accumulation and resistance mechanism in Panax notoginseng, a traditional rare medicinal herb. Chemosphere 87, 31-36.

Yang, H.-C., Fu, H.-L., Lin, Y.-F., Rosen, B.P., (2012) Chapter Twelve - Pathways of Arsenic Uptake and Efflux, in: Argüello, J.M., Lutsenko, S. (Eds.), Current Topics in Membranes. Academic Press, pp. 325-358.

Yang, Q., Tu, S., Wang, G., Liao, X., Yan, X. (2012) Effectiveness of Applying Arsenate Reducing Bacteria to Enhance Arsenic Removal From Polluted Soils by Pteris Vittata L. International Journal of Phytoremediation 14, 89-99.

Yanitch, A., Brereton, N.J.B., Gonzalez, E., Labrecque, M., Joly, S., Pitre, F.E. (2017) Transcriptomic Response of Purple Willow (Salix purpurea) to Arsenic Stress. Front Plant Sci 8, 1115.

Ye, Z.H. (2002) Vascular tissue differentiation and pattern formation in plants. Annu Rev Plant Biol 53, 183-202.

Yuan, Z., Druzhinina, I.S., Labbé, J., Redman, R., Qin, Y., Rodriguez, R., Zhang, C., Tuskan, G.A., Lin, F. (2016) Specialized Microbiome of a Halophyte and its Role in Helping Non-Host Plants to Withstand Salinity. Scientific Reports 6, 32467.

Zaady, E., Perevolotsky, A., Okon, Y. (1993) Promotion of plant growth by inoculum with aggregated and single cell suspensions of Azospirillum brasilense Cd. Soil Biology and Biochemistry 25, 819-823.

Zagorchev, L., Seal, C.E., Kranner, I., Odjakova, M. (2013) A central role for thiols in plant tolerance to abiotic stress. Int J Mol Sci 14, 7405-7432.

Zeng, Y., Guo, L.P., Chen, B.D., Hao, Z.P., Wang, J.Y., Huang, L.Q., Yang, G., Cui, X.M., Yang, L., Wu, Z.X., Chen, M.L., Zhang, Y. (2013) Arbuscular mycorrhizal symbiosis and active ingredients of medicinal plants: current research status and prospectives. Mycorrhiza 23, 253-265.

Zgadzaj, R., Garrido-Oter, R., Jensen, D.B., Koprivova, A., Schulze-Lefert, P., Radutoiu, S. (2016) Root nodule symbiosis in Lotus japonicus drives the establishment of distinctive rhizosphere, root, and nodule bacterial communities. Proceedings of the National Academy of Sciences 113, E7996-E8005.

Zhang, L., Jiang, W., Nan, J., Almqvist, J., Huang, Y. (2014) The Escherichia coli CysZ is a pH dependent sulfate transporter that can be inhibited by sulfite. Biochim Biophys Acta 1838, 1809-1816.

Zhang, N., Wang, D., Liu, Y., Li, S., Shen, Q., Zhang, R. (2014) Effects of different plant root exudates and their organic acid components on chemotaxis, biofilm formation and colonization by beneficial rhizosphere-associated bacterial strains. Plant and Soil 374, 689-700.

Zhang, S.R., Lin, H.C., Deng, L.J., Gong, G.S., Jia, Y.X., Xu, X.X., Li, T., Li, Y., Chen, H. (2013) Cadmium tolerance and accumulation characteristics of Siegesbeckia orientalis L. Ecological Engineering 51, 133-139.

Zhang, W., Cai, Y., Tu, C., Ma, L.Q. (2002) Arsenic speciation and distribution in an arsenic hyperaccumulating plant. Science of The Total Environment 300, 167-177.

Zhang, X., Zhao, F.J., Huang, Q., Williams, P.N., Sun, G.X., Zhu, Y.G. (2009) Arsenic uptake and speciation in the rootless duckweed Wolffia globosa. New Phytol 182, 421-428.

Zhang, Y., Han, X., Chen, S., Zheng, L., He, X., Liu, M., Qiao, G., Wang, Y., Zhuo, R. (2017) Selection of suitable reference genes for quantitative real-time PCR gene expression analysis in Salix matsudana under different abiotic stresses. Scientific Reports 7, 40290.

Zhao, C., Zhang, Y., Chan, Z., Chen, S., Yang, S. (2015) Insights into arsenic multi-operons expression and resistance mechanisms in Rhodopseudomonas palustris CGA009. Frontiers in Microbiology 6.

Zhao, F.J., Ma, J.F., Meharg, A.A., McGrath, S.P. (2009) Arsenic uptake and metabolism in plants. New Phytol 181, 777-794.

Zhou, S., Liu, J., Xu, M., Lv, J., Sun, N. (2015) Accumulation, availability, and uptake of heavy metals in a red soil after 22-year fertilization and cropping. Environmental Science and Pollution Research 22, 15154-15163.

Resumen

RESUMEN

I.   INTRODUCCIÓN

A pesar de que los suelos son la base y el apoyo para la vida y son un recurso no renovable que desempeña muchas funciones y brinda servicios vitales para las actividades humanas y la supervivencia del ecosistema, éstos están muy amenazados por la contaminación. Uno de los principales contaminantes químicos de los suelos son los elementos traza (ET), los cuales están presentes generalmente en concentraciones inferiores 100 mg kg^{-1}, como es el caso del cadmio (Cd), cobre (Cu), plomo (Pb), mercurio (Hg), zinc (Zn), boro (B) o arsénico (As), aunque algunos pueden alcanzar concentraciones más altas. El origen de los ET en el suelo puede ser natural (litogénico) o antropogénico (inducido por el hombre). Las actividades antropogénicas son la principal causa de la acumulación de ET en los suelos, y están relacionadas principalmente con actividades industriales y con actividades agrícolas vinculadas a la producción de fertilizantes. La empresa Nitrastur localizada en Langreo (Asturias), fue una de las principales fábricas de fertilizantes en España durante más de cincuenta años hasta su cierre en 1997 y se encuentra entre los terrenos contaminados con más altos niveles de As y Pb identificados en la región.

Algunos ET como B, Cu, Fe, manganeso (Mn) o Zn son micronutrientes esenciales para el crecimiento y desarrollo normal de la planta, mientras que otros como el Cd, Hg y el As no tienen una función biológica demostrada en las plantas, pero son absorbidos de todas maneras por éstas y causan efectos perjudiciales en la mayoría de especies vegetales. Después de su absorción y transporte a los diferentes tejidos de la planta, los iones libres de los ET ejercen su toxicidad a través de tres mecanismos principales: 1) Competición con los

elementos esenciales (debido a la similitud química) por los mismos transportadores en las células de la raíz, lo que conduce a deficiencias nutricionales y además una vez dentro de las células, puede reemplazar a iones metálicos esenciales en ciertas proteínas, interfiriendo con la actividad biológica de enzimas importantes y proteínas de señalización. 2) Unión mediante sistemas de afinidad a tioles libres u otros grupos funcionales sulfhidrilo y carboxilo de proteínas, pigmentos o enzimas importantes, lo que inhibe su función. 3) Aumento de especies reactivas de oxígeno (EOR) que causan estrés oxidativo en las células vegetales.

Por otro lado, las plantas presentan diferentes mecanismos de tolerancia basados en disminuir la concentración de iones libres de ET en sus células para prevenir los efectos dañinos descritos anteriormente y que incluyen una limitación del transporte a través de la membrana, exclusión, reducción química, quelación por glutatión (GSH), fitoquelatinas, metalotioneínas, así como la inducción de proteínas de choque térmico. Además, para hacer frente al estrés oxidativo inducido por los ET, las plantas poseen varios mecanismos para la eliminación del exceso de ERO. Estos incluyen antioxidantes enzimáticos (catalasas, peroxidasas, superóxido dismutasas ...) y no enzimáticos (GSH, algunos compuestos fenólicos, prolina, carotenoides ...).

Debido al efecto coordinado de los mecanismos mencionados anteriormente, algunas plantas pueden crecer en áreas muy contaminadas y esta propiedad puede ser aprovechada en la remediación de suelos contaminados. Este proceso se denomina fitorremediación y propone una alternativa ecológica eficaz a las costosas tecnologías de remediación fisicoquímicas. Sin embargo, la fitorremediación también tiene limitaciones, una de las más reconocidas es que, por lo general, es más lenta que otras técnicas de remediación convencionales. Sin embargo, el uso de especies leñosas, que presentan gran biomasa, junto con la utilización de los microorganismos asociados a sus raíces, puede acelerar este proceso. Además, este proceso de bioaumentación puede potenciar la salud y el crecimiento de las plantas, mejorar la absorción de agua y minerales, e incluso inhibir el crecimiento y desarrollo de microorganismos patógenos del suelo. Por lo tanto, la integración de la biorremediación microbiana y la fitorremediación es una estrategia prometedora para la remediación de contaminantes, que en combinación con la selección y caracterización de plantas acumuladoras autóctonas y sus microorganismos asociados aumenta la tasa de éxito del proceso de fitorremediación. Aunque varios trabajos han estudiado el potencial de distintas especies de sauces en fitorremediación, las investigaciones sobre su tolerancia a los contaminantes son escasas. Comprender esta tolerancia, así con las relaciones que

subyacen en la selección del microbioma por parte de la planta, permitirá el diseño de procesos efectivos de fitorremediación.

Finalmente, aunque se piensa que la colonización de bacterias es más eficiente en su huésped nativo, un punto muy interesante sería estudiar si la inoculación de bacterias aisladas de sauces a otras especies vegetales sería también eficiente en la tolerancia al As. Esto no solo permitirá profundizar en la interacción entre bacterias y plantas, sino también en ampliar la utilización de estas cepas aisladas y caracterizadas en fitorremediación.

II. PLANTEAMIENTO Y OBJETIVOS

Hasta ahora, la mayoría de los estudios descritos basados en fitorremediación se centran en estudiar de forma independiente el efecto que las comunidades bacterianas y las plantas tienen en la absorción, degradación y acumulación de contaminantes. Además, generalmente; éstos se realizan en suelos o sustratos contaminados artificialmente con un solo contaminante y con plantas modelo, lo que podría no reflejar fehacientemente los resultados obtenidos en campo, ya que es necesario tener en cuenta una amplia gama de interacciones bióticas y abióticas.

Por lo tanto, el objetivo principal de esta tesis ha sido estudiar el comportamiento de plantas inoculadas y no inoculadas con bacterias, crecidas en diferentes condiciones experimentales, desde cultivo *in vitro* hasta campo, y expuestas principalmente a As y otros contaminantes, con el fin de evaluar su idoneidad para ser utilizadas en fitorremediación. Para este objetivo, seleccionamos un clon de sauce (*Salix atrocinerea*) capaz de crecer de forma natural en un suelo contaminado con múltiples ET y se estudió su crecimiento y la acumulación de ET, tanto en plantas inoculadas como no inoculadas, así como los mecanismos genéticos y metabólicos implicados en la tolerancia al As. Para ello, se han establecido cinco objetivos parciales:

1. **Identificar y caracterizar las comunidades bacterianas asociadas a las raíces de *S. atrocinerea* que crecen en una zona industrial altamente contaminada**. Utilizando métodos de cultivo tradicionales y computacionales, se estudiaron las poblaciones bacterianas cultivables y no cultivables, y se realizó, respectivamente, una caracterización fenotípica y predictiva para la tolerancia a As, así como un estudio de las propiedades que potencian el crecimiento vegetal.

2. **Evaluar el potencial de *S. atrocinerea* para tolerar y acumular As y estudiar los mecanismos implicados en estos procesos.** A diferentes periodos de cultivo, se estudió la acumulación y especiación de As, el análisis de compuestos tiólicos y las respuestas genéticas involucradas en la tolerancia al As.

3. **Estudiar *in vitro* los efectos de la inoculación bacteriana en la acumulación y tolerancia al As en plantas de sauce.** Las bacterias previamente caracterizadas fueron ensayadas en plantas de sauce cultivadas *in vitro* y expuestas a As. Se realizó una caracterización detallada de los mecanismos fisiológicos implicados en la acumulación y tolerancia al As, y se determinó cómo la inoculación bacteriana afecta a los mismos.

4. **Caracterizar las respuestas fisiológicas de plantas inoculadas y no inoculadas cultivadas en un suelo industrial contaminado.** Las plantas inoculadas y no inoculadas se cultivaron en macetas con un sustrato control o un suelo industrial contaminado, y se estudió la interacción planta-bacteria en estos sustratos. En paralelo, se realizó un experimento de seis meses en el terreno contaminado y se hizo especial énfasis en las diferencias obtenidas entre maceta y campo en relación con la acumulación de ET y la tolerancia de las plantas a los mismos.

5. **Evaluar si las bacterias aisladas de *S. atrocinerea* pueden mejorar la tolerancia al As en otras especies vegetales.** Se investigó la acumulación de As y los cambios transcripcionales en plantas de *Arabidopsis thaliana* inoculadas con *Pantoea* sp. Además, se evaluó el papel de la inoculación bacteriana en la tolerancia al As en plantas salvajes y en el mutante *cad2-1* (deficiente en GSH).

III.   RESULTADOS Y DISCUSIÓN

La concentración total de Hg, Zn, Cu, Pb y As en el suelo de este estudio superó en 3, 6, 7, 15 y 20 veces, respectivamente, la concentración permitida para un suelo industrial. En esta tesis se estudió especialmente el As puesto que el suelo en estudio excedió con mucho su valor de referencia máximo asignado para actividades industriales. Ha sido demostrado que las bacterias asociadas a las raíces de *S. atrocinerea*, al igual que estas plantas, han desarrollado varios mecanismos para hacer frente al As que les permite tolerar altas concentraciones de este metaloide en el suelo. Sin embargo, a pesar de la alta concentración del As en el suelo, el análisis de fraccionamiento de metales (método BCR) mostró que solo una pequeña fracción del As total estaba potencialmente disponible, mientras que la mayoría de los ET están atrapados dentro de la estructura cristalina de los minerales del

suelo. A pesar de que la concentración de As disponible no representa un gran riesgo ambiental frente a la concentración total medida en el suelo, es necesaria su remediación para recuperar su valor económico. La baja biodisponibilidad de As en este suelo podría explicarse por el hecho de que, en suelos aeróbicos, como es el caso del suelo de estudio, la mayor parte del As (95%) está en forma de arseniato (As V), que es menos móvil que el arsenito (As III). Sin embargo, se sabe que algunas bacterias pueden reducir el As V a As III. Esta propiedad puede aumentar la disponibilidad del As en el suelo, incrementando su absorción por la planta y reduciendo así el tiempo necesario para la descontaminación.

La diversidad genética asociada con un rápido crecimiento y ciertas características fisiológicas y bioquímicas son requisitos importantes para la selección de genotipos útiles en fitorremediación. Por lo tanto, en este trabajo se partió de un clon de *Salix atrocinerea* seleccionado previamente por su tolerancia al As y por ser capaz de acumular altas concentraciones de As en comparación con las descritas en la literatura. Además, también se aislaron y caracterizaron las bacterias asociadas a su raíz, lo cual constituye un enfoque adecuado para mejorar la fitorremediación. Esto se debe a que tanto las plantas como las bacterias que crecen en un área históricamente contaminada se han adaptado a la presencia de los ET y ambos se benefician de esa interacción. Mediante el uso de medios de cultivo y herramientas informáticas, se observó que las interacciones entre el suelo, los microorganismos y las raíces dan forma a las comunidades bacterianas, tal y como se muestra en la composición de las diferentes bacterias observadas en el suelo, la rizosfera y la endosfera. Los criterios de selección para elegir las cepas más convenientes para mejorar la eficiencia de la fitorremediación en este estudio se basaron en la tolerancia al As, la reducción del As y la presencia de rasgos que potencian el crecimiento de las plantas. De acuerdo con esto, la bacteria gram negativa *Pantoea* sp. y la gram positiva *Rhodococcus erythropolis* fueron seleccionadas para experimentos de bioaumentación.

En un primer ensayo hidropónico con *S. atrocinerea* para certificar su tolerancia a As, la concentración de metaloide empleada se basó en la fracción disponible en el suelo de estudio. De esta manera, a una concentración de 18 mg As kg^{-1}, se describieron algunas de las respuestas fisiológicas implicadas en la tolerancia al As y que permiten su acumulación en tejidos vegetales incluso a concentraciones descritas como tóxicas en plantas no tolerantes. Después de 30 días de exposición a As, *S. atrocinerea* no mostró ningún síntoma de toxicidad y fue capaz de acumular este metaloide en sus hojas a una concentración mayor que la presente en el medio de cultivo. Además, *S. atrocinerea* también es capaz de crecer en un terreno industrial que también presenta otros ET a concentraciones tóxicas (por ejemplo, Zn, Pb). En consecuencia, la alta acumulación de As en *S. atrocinerea*, junto con su

gran biomasa, resalta el potencial del sauce para la fitorremediación de suelos con varios de estos contaminantes. Dentro de los mecanismos de tolerancia, la producción y acumulación de fitoquelatinas, compuestos quelantes naturales en plantas que se unen al elemento tóxico y lo almacenan en las vacuolas, se observó tanto en raíces como en hojas de *S. atrocinerea* como una respuesta temprana ante la exposición al As, destacando que la síntesis y la acumulación de tioles no proteicos pueden considerarse una respuesta que ayuda a las plantas a restablecer su homeostasis y progresar en sus etapas de desarrollo al unirse al As y limitar su toxicidad. Estos resultados coinciden con la regulación transcripcional de los transportadores relacionados con el As, reductasas de As, glutatión sintetasa y metalotioneínas.

Dado que la absorción del As por la planta depende de la concentración total de As disponible en su entorno y, lo que es más importante, de la especiación del mismo en el suelo o en el medio de cultivo, las plantas de sauce se inocularon con las bacterias seleccionadas y se observó cómo la especiación de As (hasta el 10% de As III con *Pantoea* sp. y el 90% con *R. erythropolis* frente al 4% en tratamientos no inoculados) afectó a la acumulación de As, síntesis de compuestos quelantes y expresión génica. Mientras que la inoculación con *Pantoea* sp. incrementó la absorción de As por la planta, la inoculación con *R. erythropolis* aumentó la toxicidad del mismo hasta un umbral que disminuyó la tolerancia de la planta y que además resultó en una reducción de la acumulación del mismo. El aumento en la peroxidación lipídica solo en plantas expuestas al As e inoculadas con *R. erythropolis*, indicó que la toxicidad del As está relacionada con su especiación, y mostró la ausencia de daño en las membranas celulares al menos que altas concentraciones de As III estuvieran presentes en hojas. Además de alterar la expresión génica de transcritos relacionados con proteínas involucradas en el transporte, la reducción química, la quelación y el secuestro del As, la inoculación bacteriana causó una sobreexpresión de transcritos relacionados con los flavonoides, que también coincidió con un aumento de antocianinas, taninos condensados y fenoles totales en comparación con plantas no inoculadas. Esto no es inesperado ya que los flavonoides han sido descritos como moléculas de señalización cruciales en las interacciones planta-bacteria. Esta alta biosíntesis de taninos bajo inoculación bacteriana es interesante, ya que un incremento en la concentración de taninos en las hojas puede conferir a las plantas una ventaja al reducir la depredación de artrópodos en condiciones de campo.

Los ensayos de campo a menudo están expuestos a una gran cantidad de factores variables, como la distribución irregular del pH del suelo, los contaminantes, los nutrientes o el agua, entre otros, que pueden afectar las respuestas de las plantas a los tratamientos

experimentales. En este trabajo, al comparar un experimento en maceta con un experimento en campo utilizando el mismo suelo, mostró cómo la influencia de distintos factores en campo origina resultados divergentes tanto en crecimiento como en acumulación de ET entre plantas inoculadas y no inoculadas. Dado que el crecimiento bacteriano aumenta al incrementar el pH, en este estudio, el suelo utilizado en maceta se mezcló de forma homogénea, mientras que en campo las plantas se colocaron al azar, lo cual podría justificar algunas de las diferencias encontradas entre el efecto de la inoculación bacteriana en maceta y en campo. Además, también se observó cómo las plantas de *S. atrocinerea* utilizadas en esta tesis, presentaron una reducción del crecimiento y biomasa en comparación con aquellas plantas que crecen en un sustrato control. Esto indica que la presencia de otros contaminantes, además del As, a altas concentraciones y la interferencia de los mismos, reducen la tolerancia de *S. atrocinerea* para crecer en un suelo contaminado con múltiples ET.

La bioaumentación mediante la inoculación de *Pantoea* sp. y *R. erythropolis* en macetas con el suelo contaminado disminuyó las concentraciones de ET (mg de metal(oide) kg^{-1} planta PS) en raíces y hojas de plantas de *S. atrocinerea*. Sin embargo, se observó una tendencia opuesta en el ensayo de campo. En las macetas, la inoculación bacteriana incrementó la biomasa de *S. atrocinerea*, probablemente al disminuir la absorción de los ET por la planta. En consecuencia, la cantidad total de los ET extraídos por las plantas inoculadas no fue significativamente diferente de las plantas no inoculadas y, puesto que la biomasa es mayor, a largo plazo, las plantas inoculadas podrían extraer una cantidad mayor de ET que las plantas no inoculadas. En campo, la inoculación con *Pantoea* sp. llevó a concentraciones más altas de ET en las hojas y una mayor toxicidad para la planta que causó una reducción de la biomasa y, por lo tanto, se extrajeron menos Cu, Zn y Cd en comparación con las plantas no inoculadas. Por el contrario, aunque la inoculación con *R. erythropolis* en campo originó concentraciones de ET más bajas que con *Pantoea* sp., estas plantas presentaron una mayor fitoextracción de ET debido a un incremento de la biomasa vegetal.

En este estudio, como ya se mencionó anteriormente, el pH podría ser un factor importante para explicar las diferencias observadas entre maceta y campo, aunque esto también podría atribuirse a diferencias en los exudados de la raíz de las plantas en diferentes condiciones experimentales y/o a las concentraciones o disponibilidad de metabolitos microbianos y nutrientes del suelo y a la influencia de las condiciones climáticas, así como a una disminución del inoculante bacteriano debido a su lixiviación en campo. Además, una mayor acumulación de ET en maceta que en campo puede ser debida a un volumen más restringido de suelo que las raíces pueden explorar, y por lo tanto la

mejor absorción de iones metálicos en maceta. En conjunto, estos resultados resaltan la complejidad de las interacciones suelo-microorganismos-planta y la necesidad de más estudios en esta dirección. Además, a pesar de un patrón de expresión génica contradictorio, los taninos condensados fueron nuevamente más altos en las plantas inoculadas tanto en el suelo control como en el contaminado, y se observó lo mismo en campo, resaltando el potencial biocatalizador de las bacterias asociadas a la raíz.

La posible aplicación de las bacterias aisladas de *S. atrocinerea* puede cruzar la barrera entre especies como se observó mediante la inoculación de *Pantoea* sp. aislada de *S. atrocinerea* a *Arabidopsis thaliana*. Al comparar la expresión génica del mutante de tipo salvaje (WT) y *cad2-1* (deficiente en GSH), en condiciones no inoculadas e inoculadas con *Pantoea* sp., se demostró que la inoculación bacteriana aumenta la acumulación del As en las raíces de las plantas WT. Sin embargo, una mayor presencia de As III en las raíces de ambos genotipos causó una diferente respuesta de translocación, basada en la sensibilidad del genotipo al As: mayor crecimiento y acumulación del As en el WT y disminución del crecimiento y de la acumulación del As en el mutante *cad2-1*. Estos cambios estaban acompañados por una regulación diferencial de los genes implicados en las respuestas de absorción y deficiencia de fosfato. Así como por una disminución de la regulación del transportador de arsenito *NIP7; 1*, de la inducción en la expresión de genes relacionados con el metabolismo del azufre (incluidas las metalotioneínas *MT1a* y *MT2a* solo en el mutante *cad2-1*) y una regulación diferencial de los genes relacionados con el estrés oxidativo.

Los estudios realizados en este trabajo, incluidas las respuestas diferenciales en las diversas condiciones experimentales, han demostrado, en primer lugar, la necesidad crucial de comprender a nivel molecular y biogeoquímico, los mecanismos que subyacen en la interacción sustrato-microorganismo-planta; y en segundo lugar, que su aplicación en la fitorremediación, así como en otros procesos biotecnológicos, implica una correcta selección de bacterias y plantas, ambas adecuadas a las necesidades y objetivos específicos en cada caso.

IV.   CONCLUSIONES

Las principales conclusiones de esta tesis son las siguientes:

1. Las interacciones entre las raíces, los microorganismos y el suelo tienen un efecto en el microbioma de la raíz y explican las diferencias en la composición y diversidad de las comunidades bacterianas del suelo, la rizosfera y la endosfera de *S. atrocinerea*.

2. La presencia de diversos caracteres que potencian el crecimiento vegetal (producción de hormonas, sideróforos, fijación de nitrógeno ...) en las bacterias asociadas a la raíz de *S. atrocinerea*, indica que las relaciones a nivel celular entre las bacterias y su huésped influyen en la acumulación y tolerancia a los contaminantes por la planta. Lo cual aporta nuevas posibilidades para favorecer, a través de la bioaumentación, a las plantas bajo condiciones de estrés crónico.

3. Las plantas de sauce que crecen en presencia de arseniato en condiciones de hidroponía, muestran una regulación de los genes involucrados en respuestas al estrés, reducción de arseniato, transportadores de nutrientes, síntesis de glutatión y en el secuestro de As en las vacuolas, lo que coincide con una rápida presencia y acumulación de arsenito en tejidos radiculares, un perfil nutricional alterado y la síntesis *de novo* y aumento de compuestos tiólicos no proteicos que contribuyen a la tolerancia de la planta al metaloide.

4. En condiciones *in vitro*, se demostró cómo la absorción de As por parte de la planta está influida por la concentración de As disponible en el medio de cultivo y, lo que es más importante, por su estado de especiación; además también se ve afectado por la inoculación bacteriana. Por tanto, la inoculación de plantas con *Pantoea* sp. o *R. erytrhopolis* constituye un mecanismo sinérgico que altera la toxicidad del As al cambiar su especiación, movilidad y acumulación en *S. atrocinerea*.

5. En maceta, la reducida acumulación de contaminantes obtenida en plantas inoculadas con *Pantoea* sp. o *R. erythropolis* y cultivadas en un suelo contaminado dio lugar a una mayor biomasa en comparación con las plantas no inoculadas, que a largo plazo podría inducir un aumento en la fitoextracción de contaminantes.

6. En campo, la inoculación bacteriana resultó en una mayor acumulación de As y Pb en la planta y, por tanto, en una mayor fitoextracción, junto a una elevada translocación de Zn. Mientras que en las plantas inoculadas con *Pantoea* sp. se observó una mayor acumulación de metal(oid)es y una disminución de la biomasa, en aquellas inoculadas con *R. erythropolis* se apreció el efecto contrario.

7. Dada la acumulación de Zn y Cd en las raíces de sauce y sus tasas de translocación, junto con la acumulación de As y Pb en las raíces y la capacidad para crecer en terrenos contaminados, *S. atrocinerea* es una especie adecuada para ser utilizada en fitorremediación. Además, la bioaumentación con *Pantoea* sp. o *R. erythropolis* puede reducir el tiempo necesario para la remediación del suelo.

8. Teniendo en cuenta que los resultados obtenidos en el laboratorio no siempre pueden extrapolarse a campo, se deduce la necesidad de realizar ensayos de campo para obtener resultados exitosos en fitorremediación. Sin embargo, a partir de estudios controlados se ha obtenido un conocimiento considerable sobre la tolerancia al As, los procesos de remediación basados en bioaumentación y la interacción planta-bacteria.

9. Las bacterias seleccionadas han mostrado un gran potencial en fitorremediación, no solo por su capacidad para transformar y neutralizar y alterar la toxicidad de contaminantes como el As, sino también por su potencial para sintetizar enzimas como las fitasas o arseniato reductasas como se muestra en su genoma bacteriano. Además, las bacterias fueron capaces de inducir la acumulación de ciertas moléculas de interés en plantas como los taninos u hormonas, lo que resalta su potencial biocatalizador.

10. El uso de bacterias aisladas de *S. atrocinerea* que crecen en suelos contaminados con As también puede aumentar la acumulación y la tolerancia al As en otras especies vegetales, como se demuestra en *Arabidopsis thaliana*. Además, la inoculación de bacterias entre diferentes especies ofrece más información sobre los mecanismos implicados en la interacción bacteria y planta, y que es necesaria para adaptar estrategias específicas de remediación de contaminantes por medio del binomio planta-bacteria.